THE ROLE OF MAST CELLS IN RAPID EXPULSION OF *TRICHINELLA SPIRALIS* IN THE RAT

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The aim of this research was to test the hypothesis that immune complexes activate mucosal mast cells to cause rapid expulsion of the *Trichinella spiralis* from the rat intestine. Rapid expulsion is a phenomenon in the rat that is characterized by nearly complete elimination of a secondary infection. To test this hypothesis, mast cells were cultured from bone marrow precursors. These cells have a homogenous mucosal phenotype and were compared with the mucosal mast cell line, RBL-2H3 cells. Each cell type was assayed for binding and activation by parasite specific immune complexes. The results showed that IgE- and IgG2a-containing immune complexes bound and activated RBL-2H3 cells, but only IgE significantly activated bone marrow-derived mast cells (BMMC). IgG1 and IgG2b immune complexes bound both cells types but did not trigger degranulation. IgG2c did not bind mast cells. Because IgG2a-containing complexes activated RBL-2H3 cells *in vitro* and this isotype differed from other isotypes in its mechanism for induction of rapid expulsion, we investigated whether this isotype might activate mast cells *in vivo*. Rats infected with *Heligmosomoides polygyrus* then passively immunized with IgG2a demonstrated rapid expulsion without significant mucus entrapment of larvae. IgG2a-mediated protection was complement independent and Fc-dependent; however, no significant increase in RMCP-II release was detected during expulsion. These results are consistent with a

role for other FcR-bearing cells in IgG2a-mediated protection. In a parallel investigation, confounding inflammatory factors induced by oral infection were eliminated by assaying rapid expulsion induced by parenteral infection. Protection conferred by parenteral immunization was as potent as that induced by natural infection was more robust than the *H. polygyrus* model. Parenterally infected rats did not display intestinal mastocytosis, eosinophilia, or goblet cell hyperplasia; however, mast cells were activated and mucus entrapment of larvae occurred at levels comparable to those observed in rats infected naturally. Complement was not necessary for protection mediated by parenteral immunization.

BIOGRAPHICAL SKETCH

Seana Michele Thrasher was born in Fayetteville, North Carolina on March 13, 1970. She attended high school in Richardson, Texas then moved to Holsby Brunn, Sweden for a year of divinity education at the Torchbearers International School. At age eighteen, she began a Bachelor of Science in Biochemistry at the University of Georgia in Athens, Georgia. After obtaining her B.S., she attended Colorado State University in Fort Collins, Colorado as a graduate student in Biochemistry. For personal reasons, she postponed her Ph.D. studies and started veterinary school at the University of Georgia. Upon completion of her D.V.M., she moved to Guelph, Ontario in Canada and pursued internship training in Large Animal Medicine and Surgery at Ontario Veterinary College. She entered the Graduate Field of Comparative Biomedical Sciences at Cornell University in Ithaca, New York for study culminating in a Doctor of Philosophy.

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CHAPTER ONE

Introduction

A. Relevance of this research

Prevalence of allergic diseases is increasing worldwide, most markedly in Western and English speaking countries (16). In the United States, asthma is considered epidemic (80). In addition to increased incidence of Th2 mediated disease, including asthma, atopic eczema, and allergic rhinitis, there is increased incidence of Th1 mediated autoimmune disease, such as diabetes, inflammatory bowel disease, and multiple sclerosis (35, 96, 103). While the reasons for this are multi-factorial, it is clear that there is a direct correlation between reduced infection and increased allergic disease. This association was first recognized by Strachan in the formulation of the hygiene hypothesis (108). Emergence does not correlate well with pollution (29), but more with factors related to hygiene such as decreased parasite burdens and reduced exposure to infectious agents in early childhood (70, 117, 125). Infections appear to modulate the immune system, most likely through T regulatory cells that inhibit activation of T effector cells and limit inflammation, thereby preventing manifestations of allergy and autoimmunity (41, 96).

Both allergic disease and helminth infections are characterized by Th2 responses, but the former is the product of inappropriate immune regulation. It has been observed that parasitic infections can decrease or prevent allergic responses. For example, the suppressive effect of parasitic infection on atopy was demonstrated in persistently parasitized children in Gabon. Children treated with anthelmintic showed stronger skin prick test responses to house dust mite antigen provocation compared to untreated children (117). One hypothesis for decreased allergic sensitivity in parasitized individuals states that IgE binding sites on basophils and mast cells are blocked by polyclonal

IgE induced by parasitic infection. However, changes in total serum IgE concentrations in Gabonese children were not sufficient to implicate this as the mechanism for increased allergic sensitivity (117). This hypothesis was further refuted by *in vitro* studies demonstrating that polyclonal IgE from filarial-infected individuals does not saturate basophil Fc receptors and only blocks allergen-specific IgE responses at non-physiologic concentrations (82). Recent data support a role for T regulatory cells in allergy observed in persistently parasitized individuals.

Many allergic disorders are associated with mast cell activation at mucosal surfaces. Likewise, gastrointestinal nematodes induce mastocytosis and for some parasites, mastocytosis is necessary for parasite expulsion (66, 126). In addition, parasitic infection induces production of IL-10 and TGF-β, two canonical regulatory cytokines (123). Since humans have evolved in close association with parasites, it is reasonable to predict that removal of such a persistent stimulus to the immune system may affect immune responses to other antigens. Investigations of the immune response to parasites could be useful for discovering methods to control immune pathologies seen in the absence of parasitism.

In developed societies, the absence of parasitic infection likely contributes to increases in allergic and autoimmune diseases; however, in developing societies parasitic nematode infections remain a significant health problem for people and for livestock. Of particular concern is the ability of parasites to become resistant to anthelmintic treatments. This concern is augmented by a dearth of new anthelmintic drugs. In 2001, the World Health Organization (WHO) set minimal treatment targets for reducing morbidity due to helminth infections in humans (5). Likewise, the Global Alliance for the

Elimination of Lymphatic Filariasis (GAELF) organized a two-drug (albendazole and ivermectin) yearly treatment plan for 26 million people in 22 endemic countries (5). As these mass treatment programs become more frequent, the emergence of multi-drug resistance in human parasites may rival multi-drug resistance documented in livestock populations (46, 61).

There are fewer case reports of anthelmintic drug resistance in parasites of humans than in those of livestock. However, resistance has been documented for treatment with praziquantel of *Schistosoma haematobium* (8), ivermectin of *Onchocerca volvulus* (43), and pyrantel of *Ancylostoma duodenale* (99). Treatment programs for gastrointestinal parasites of livestock have resulted in multi-drug resistance on a global scale, particularly in small ruminant populations (46). In goat populations of the southern United States, gastrointestinal nematodes are resistant to all currently available anthelmintics (84). Ivermectin resistance presents ever increasing treatment complications not only for *Haemonchus contortus* in small ruminants but also for *Onchocerca volvulus* in humans (43). In light of these trends, vaccination against parasitic infection has renewed relevance for both human and livestock populations.

The goal of vaccine development against parasites has been encumbered by the complexity of immune responses to parasitic infection compounded by mechanistic differences among the large number of host-parasite interactions. While there certainly is no single path to solving this complex issue, our understanding of immunity to parasites has advanced using model systems such as vaccination against *H. contortus* in sheep (98). Vaccination against *Onchocera ochengi* in cattle induced protective immunity (110), and this model might be beneficial for vaccine development against filarial nematodes of humans. Another productive laboratory model for

studying the immune response to parasitic nematode infection is *Trichinella spiralis* in rats.

B. Life-cycle of *T. spiralis*

T. spiralis is a useful laboratory model of nematode infection for several reasons. First, *T. spiralis* is unusual among parasitic nematodes in that it completes its life-cycle within one host. Thus the parasite is easily propagated and maintained within the laboratory environment. Second, during the course of the *T. spiralis* life cycle, the first-stage larvae resides in the mucosal environment of the gastrointestinal tract as well as within the systemic environment of the skeletal muscle. This provides an opportunity to test mucosal and systemic immunity generated against the same life stage.

Infectious first-stage larvae (L1) are transmitted to the host by ingestion of raw meat from an infected animal. This initiates the enteral phase of infection. Digestion by stomach pepsin releases larvae from muscle tissue. L1 immediately enter the epithelium of the proximal small intestine. Within thirty hours the larvae molt four times to reach the adult stage. Mating occurs within the intestinal epithelium. Female worms are viviparous and release live newborn larvae (NBL) which migrate through the portal circulation and vasculature. Migrating larvae pass through most organs of the body, including the heart, liver, and lungs. NBL have a predilection for skeletal muscle where they begin establishment of their niche within a structure called the Nurse cell. For rodents, Nurse cell development is concurrent with expulsion of adult worms from the intestine, between ten and twenty days after oral infection. Thus ends the enteral phase of infection and begins the parenteral or systemic

phase of infection which is a chronic, persistent infection eliciting a potent immune response. After approximately twenty days of development in the Nurse cell, the larvae become infectious, and the life-cycle can be repeated in a new host. The Nurse cell can support viable larvae for as long as 30 years.

It is important to note the distinction between the enteral and parenteral phases of *T. spiralis* infection. In some of our studies, we manipulated the life-cycle by establishing muscle infections without enteral infection by injecting rats intravenously with NBL.

C. The immune response to *T. spiralis*

1. Rapid expulsion of T. spiralis

Rats naturally infected with *T. spiralis* are completely immune to secondary infections. This strong immune response has been referred to as rapid expulsion. Rats respond to a challenge infection with *Trichinella* by expelling 99% of intestinal larvae within a few hours (23, 33, 71, 76). This protective immunity lasts seven weeks or longer in rats (6). Multiple rat strains display rapid expulsion when challenged after natural infection (18). Because this immunity is exclusively observed in rats (6), mechanistic studies have been hindered due to the lack of transgenic and knock-out animals as well as reagents that are more readily obtainable in mice. Nevertheless, studies of rapid expulsion in the rat provide insights to immunity against parasites.

Historically, investigations have attempted to identify a single mediator of rapid expulsion, but this has been challenging because of the complexity of the inflammatory response to oral infection that is induced prior to expulsion. In adult rats, it was determined that rapid expulsion requires two discrete

stimuli (20): both intestinal inflammation and antibody. In addition, rapid expulsion is specific for first-stage larvae (L1) (22) and can be recreated by passive immunization with parasite-specific IgE or certain IgG isotypes. However, passive immunity occurs only in recipients that have previously experienced an oral infection with a heterologous nematode, such as *Heligmosomoides polygyrus* or *Nippostrongylus brasiliensis* (20, 51). Since antibody alone is not protective, it has been speculated that antibodies might be interacting with some cellular component of the immune system that is expanded or induced by intestinal infection. Prominent cellular changes in the intestine due to infection include mastocytosis, eosinophilia, and goblet cell hyperplasia (6, 60). The first two cell types bear Fc receptors (FcR), and it is possible that immune complexes might activate these cells in the inflamed intestine.

While a direct role for mast cells in rapid expulsion has not been demonstrated, intestinal mastocytosis and mast cell activation occurs during expulsion of adult worms from the intestine in a primary infection (124), and mast cells are activated during expulsion of a challenge infection (83). Additionally, mediators released from mast cells during expulsion result in dramatic changes in the intestinal environment which may be detrimental to parasite establishment. Rat mast cell protease-II (RMCP-II) (105, 106), prostaglandins (128), serotonin (54, 75), leukotrienes (83), histamine (54, 128), and cytokines (54) affect intestinal secretion (34), permeability (25, 102), and smooth muscle contraction (119). Any one or more of these mechanisms could contribute to nematode expulsion. Since nematode infection provokes such complex intestinal changes, a single mechanistic explanation for rapid expulsion remains difficult to elucidate. Furthermore, infection also induces

$CD4^+$ T cell activation, $CD8^+$ T cell activation, B cell activation, and specific antibody production. Clearly, the immunity referred to as rapid expulsion is a complex orchestration of events leading to parasite elimination from the intestine. Any of these factors could mediate rapid expulsion or cooperate to produce it. The role of each mediator in primary and secondary infections will be discussed individually.

2. The role of mast cells in immunity to T. spiralis

Mast cells play a role in primary expulsion of *T. spiralis* from mice and rats. *T. spiralis* induces intestinal mastocytosis, and mast cell activation coincides with expulsion of adult worms from the intestine of both mice and rats (6). Intestinal mastocytosis is T-cell and antigen dependent (94, 95, 100). Expulsion of adult worms is prolonged in athymic nude rats (120), and mast cell deficient Wv/Wv mice infected with *T. spiralis* are slower to expel adult worms than mast cell-replet littermates (50). In mice, bone marrow grafting of mast cells resulted in restoration of intestinal mastocytosis and accelerated expulsion (50, 90). Stem cell factor (SCF) and interleukin-3 (IL-3) are essential for development of intestinal mastocytosis to nematode infections. Expulsion of adult *T. spiralis* is impaired in mice deficient in either IL-3 or SCF or both (69). Neutralization of SCF by injecting mice with anti-SCF or anti-SCF receptor antibodies impaired intestinal mastocytosis and adult worm expulsion of *T. spiralis* (39, 47). Conversely, supplementation of mice with IL-3 resulted in an earlier induction of intestinal mastocytosis and enhanced worm expulsion of *T. spiralis* (68). Additionally it has been observed that interleukin-9 (IL-9) transgenic mice have abnormally large populations of intestinal mast cells and displayed enhanced expulsion of adult *T. spiralis*

worms (45). This enhanced expulsion can be abrogated by treatment with antibody against the SCF receptor, c-kit (45). It has been observed that mouse strains demonstrate different kinetics for *T. spiralis* adult worm expulsion, and these differences correlate with mastocytosis within the intestine (28). Mast cell activation occurs during adult worm expulsion, as demonstrated by detection of mouse mast cell protease-I (MMCP-I) in sera (57). Furthermore, *T. spiralis* expulsion is delayed in mice lacking MMCP-I (66).

Intestinal mastocytosis occurs during enteral infection of rats with *T. spiralis* (75). Mast cell activation is coincident with rapid expulsion of *T. spiralis* from the rat intestine (20, 83). Although pharmacologic inhibition of many mast cell mediators released during expulsion failed to inhibit rapid expulsion (24), inhibition of serotonin by the serotonin receptor antagonists, ketanserin and MDL-722222, significantly inhibited rapid expulsion (127). These findings are discussed in more detail in section 3 below.

Because rapid expulsion is a phenomenon unique to the rat, studies implicating the mast cells in rapid expulsion have been limited due to the shortage of mutant, knock-out, or transgenic animals. A spontaneous mutant rat strain, Ws/Ws, has been described that is mast cell deficient (111). There are very few reports describing immunity in this strain, largely due to their poor reproductive performance and overall health status.

The role of the mast cell in immunity to parasitic nematodes has been demonstrated for *Strongyloides venezuelensis*. Similar to *T. spiralis* infection, Wv/Wv mast cell deficient mice infected with *S. venezuelensis* are slower to expel adult worms; and bone-marrow grafting of mast cells restores intestinal mastocytosis and accelerates expulsion (63). In contrast, immunity to some

parasitic nematodes does not require mast cells. Expulsion of *Nippostrongylus brasiliensis* is not dependent on intestinal mastocytosis (1, 112). Thus the influence of mast cells in immunity to parasitic nematodes varies among species.

3. The role of increased intestinal permeability and secretion in immunity to T. spiralis

Mast cells release many pro-inflammatory mediators that cause dramatic physiologic changes in the intestinal environment. Experiments have been designed to remove or replace these mediators in order to identify which one might be responsible for worm expulsion. Increased intestinal permeability and secretion occurs during expulsion of primary infections with *T. spiralis* and is thought to contribute to parasite elimination from the intestine. Treatment of IL-9 transgenic mice with antibody against the SCF receptor, c-kit, decreased intestinal permeability and delayed expulsion of *T. spiralis* adult worms (77). This suggests that mast cell induced intestinal permeability contributes to worm expulsion.

Intestinal permeability is increased during secondary infection with *T. spiralis*, and some studies implicated increased intestinal secretion and permeability in parasite expulsion. MMCP-I deficient mice displayed reduced intestinal permeability and delayed expulsion of a challenge infection (77). Increased epithelial permeability via a paracellular route has been demonstrated during a challenge infection with *N. brasiliensis* in the rat and is due to RMCP-II (106). RMCP-II alters the distribution of tight junction proteins, ZO-1 and occludin, in the intestinal epithelium leading to increased permeability (104). Since RMCP-II is released during a challenge infection

with *T. spiralis* in the rat (83), these physiologic changes in the intestine are presumably present during expulsion. Additional mast cell mediators, such as histamine, 5-hydroxy-tryptamine (5-HT), and prostaglandin E_2 (PGE_2) all contribute to anaphylaxis-induced ion transport changes in the small intestines of rats immunized against *T. spiralis* (34). These changes in intestinal Cl^- secretion are biphasic, comprised of a phase I induced by 5-HT and histamine and a phase II induced by PGE_2 (34) and mediated in part by cAMP (53, 101). This biphasic pattern of epithelial Cl^- secretion is observed in the jejunum from rats infected with *T. spiralis* and then challenged (51). Furthermore, investigations showed that rats passively immunized with immune serum exhibit only the late phase of secretion and were not protected, implicating the early response as essential for protection. Furthermore, greater numbers of mast cells or altered activation of mast cells in the mucosa appear necessary to generate a full anaphylactic response in the epithelium (51). Inhibition of histamine and prostaglandins does not affect worm expulsion (24, 127); however, inhibition of 5-HT does impede worm expulsion during challenge infection. It was demonstrated that the effects of 5-HT on worm expulsion was independent of Cl^- secretion, and that intestinal secretion was coincidental with but not contributory to expulsion (127). Other effects of 5-HT on the intestine include enhanced mucus output, increased motility, and altered mesenteric blood flow (91); and any of these might impact worm establishment within the intestine.

4. The role of muscle hypertrophy and hypercontractility in immunity to T. spiralis

A primary infection with *T. spiralis* cause changes in rat intestinal myoelectric activity resulting in abnormal gut motility that possibly contributes to parasite expulsion (93). In rats infected with *T. spiralis*, mast cell numbers were increased in the longitudinal muscle of the jejunum. Exposure to *T. spiralis* antigen induced mast cell degranulation causing muscle contraction that could be inhibited with either the mast cell stabilizer, doxantrazole, or with 5-HT antagonist, cyproheptadine (119) (75). *T. spiralis* infection in both rats and mice causes persistent hyperplasia and hypertrophy of smooth muscle in the small intestine (26, 113, 122). These smooth muscle changes alter intestinal motility and may contribute to parasite expulsion. Because nematode infections induce significant mastocytosis and 5-HT release, both of which contribute to parasite expulsion and to increased muscle contractility, it is speculated that mast cells might be mediating smooth muscle changes via 5-HT. However, experiments using mast cell deficient mice (Wv/Wv) demonstrated that this was not the case (114). While *T. spiralis* infected Wv/Wv mice display delayed expulsion that was partially restored by mast cell reconstitution, the disruptions in smooth muscle contraction are not restored (114). Wv/Wv mice also lack a population of c-kit dependent cells, interstitial cells of Cajal (ICC), which regulate the myoelectrical slow wave activity that controls intestinal motility (114). Thus, these cells promote smooth muscle contractility and likely contribute to parasite expulsion.

Additional experiments have revealed the T-cell dependency of smooth muscle hypertrophy and hypercontractility in response to *T. spiralis* infection in mice and rats (118), (115). Studies, utilizing IL-4 deficient and signal

transducer and activator of transcription factor 6 (STAT-6) deficient mice, demonstrated that acute infection with *T. spiralis* leads to increases in IL-4 and IL-13 via STAT-6 that mediate smooth muscle hypercontractility and parasite expulsion (3, 64). Smooth muscle hypertrophy and hypercontractility persists for six weeks after intestinal inflammation has abated. This persistence is due to acute changes in the smooth muscle induced by IL-4, IL-13, and TGF-β and is maintained by TGF-β. Induction of COX-2 by TGF-β leads to PGE_2 production at the level of the muscle cell (4). Rapid expulsion is demonstrated by rats challenged several months after primary infection. It is not clear that smooth muscle changes induced by primary infection in the rat are sustained in the longer term.

5. The role of eosinophils in immunity to T. spiralis

Eosinophils kill *T. spiralis* NBL when incubated in the presence of immune serum (62). It has been shown that mice treated with anti-eosinophil serum acquire increased muscle larvae burdens (48), suggesting that eosinophils participate in defense against migrating NBL. Furthermore, CCR3 deficient mice display reduced eosinophil recruitment to muscle tissue and a corresponding reduction in larvae toxicity (49). In contrast to these studies, transgenic mice overexpressing IL-5 with marked eosinophilia did not display increased larval killing (56).

In response to a primary infection with *T. spiralis*, rats mount a peripherial blood, intestinal, and bone-marrow eosinophilia (60) (15, 38, 107). However, interleukin-5 deficient mice infected with *T. spiralis* expel adult worms with kinetics similar to infected heterozygous controls, suggesting that eosinophilia does not play a significant role in expulsion of adult worms from

the intestine. Additionally, depletion of eosinophils by anit-IL-5 antibody treatment of mice infected with *T. spiralis* demonstrated no effect on the course of primary infection (55). Transgenic mice overexpressing IL-5 also did not expel *T. spiralis* any faster than control mice (56). Therefore, eosinophils more likely participate in protection against *T. spiralis* NBL than against adult worms.

A role for eosinophils in immunity to secondary infections was demonstrated in IL-5 deficient mice challenged with *T. spiralis* (116). These mice have significantly larger worm burdens and display slower expulsion kinetics. Adoptive transfer of thoracic duct T cells ($OX8^-$,$OX22^-$) from *T. spiralis*-infected rats induced intestinal eosinophilia and provided moderate protection against challenge (121). More definitive studies addressing the role of eosinophils in rapid expulsion of *T. spiralis* from the rat are lacking. Peripherial eosinophilia is associated with the expulsion of a challenge infection of *S. venezuelensis* in rats (14); however, in *N. brasiliensis* infected rats, an intense peripherial eosinophilia is mounted in the absence of intestinal eosinophilia (109). The *in vivo* function of eosinophils in protection against parasites remains controversial (17, 79).

6. The role of goblet cell hyperplasia and mucus entrapment in immunity to T. spiralis

Intestinal goblet cell hyperplasia is a characteristic response to nematode infection in the mice and rats (6). In rats, goblet cell hyperplasia and alteration in goblet cell mucins seem to play a role in immunity to *N. brasiliensis* (59, 81). Quantitative as well as qualitative changes in mucus, such as changes from neutral to acid mucins (67) and alteration in the terminal

sugars of mucins (59), enhance immunity to nematodes. Furthermore, RELMβ, a protein produced by goblet cells, is upregulated in mice infected with *T. spiralis* (65). While RELMβ binds to the chemosensory apparatus and inhibits chemotaxic function of *Trichuris muris in vitro* (13), it does not inhibit *T. spiralis* epithelial penetration and migration *in vitro* (unpublished data). Mucus entrapment alone, or in coordination with antibody (31) occurs during rapid expulsion of *T. spiralis* in the rat. In suckling rats, IgG1 and IgG2c promote entrapment of larvae in mucus (30). Mucus entrapment of larvae did not occur in weaned rats passively immunized with IgG2c nor were these rats protected (92); however *H. polygyrus* infected adult rats that were passively immunized with IgG1 and challenged did display mucus entrapment (20). Rapid expulsion does occur without mucus entrapment in rats that are immunized with an abbreviated oral infection (19). Furthermore, it has been observed that mucus entrapment is reversible and thus may not be essential for rapid expulsion of *T. spiralis* in the rat (92).

7. The role of antibody in immunity to T. spiralis

Rodents infected with *T. spiralis* mount a strong antibody response to muscle larvae. The isotypes that are prominent in the response are those influenced by Th2 cytokine activation. In general, mice respond to primary infection by producing IgE and IgG1, some IgM and IgA, and little IgG2b and IgG3 (7). Re-infection stimulates both IgE and IgG1 (85). In rats nine weeks post-infection, Th2 driven isotypes (IgG1 and IgG2a) are dominant, followed by IgG2c with little IgG2b (12). While there are conflicting data concerning the role for antibody in adult worm expulsion, there is substantial evidence that antibody is pivotal for rapid expulsion of a challenge infection (9, 12, 20). Rat

pups suckling a *T. spiralis* infected foster dam demonstrate rapid expulsion of *T. spiralis*. Immunity is conferred by parasite specific antibodies in the milk or by passive transfer of immune serum (10, 11). These antibodies are specific for a highly immunogenic sugar, tyvelose that is produced by infectious first-stage larvae (40). In addition, anti-tyvelose monoclonal antibodies IgG1 and IgG2c are protective in neonatal rats but IgG2a and IgG2b are not (9, 12, 32). While these data provide strong evidence for the role of antibody in suckling rats, the situation in weaned and adult rats differs.

Passive transfer of monoclonal antibodies or immune serum from *T. spiralis* infected rats fails to promote rapid expulsion in weaned rats (92). However, antibodies are protective when rats have been primed by an intestinal infection with an antigenically unrelated nematode such as *H. polygyrus* (20) or *N. brasiliensis* (53). IgE, IgG1, IgG2a, and IgG2c are all protective (20). Alternatively, rapid expulsion is demonstrated by rats infected with *H. polygyrus* (21), or *T. spiralis* (34) and immunized parenterally with *T. spiralis* larval antigens.

Whether or not antibody cooperates with complement to cause rapid expulsion has not been determined. All rat IgG isotypes can fix complement; however, IgG2a and IgG2b activate complement most efficiently. Bell et al. showed that depletion of complement with cobra venom factor inhibited rapid expulsion; however, the result was not repeatable (24). Since antibody alone cannot cause rapid expulsion, it has been thought that antibodies might be interacting with FcR-bearing immune cells, with complement, or with intestinal mucus to effect rapid expulsion.

8. The role of lymphocytes in rapid expulsion of T. spiralis

A role for lymphocytes in expulsion has not been thoroughly investigated. Furthermore, it is difficult to interpret the effects of immunosuppression in rats, because T-cell suppression decreases intestinal mastocytosis, eosinophilia, and antibody responses of which may contribute to expulsion (52). Transfer of thoracic duct lymphocytes, both B and T cell fractions, conferred protection to naïve rats (36, 37, 121); however, this immunity was not rapid expulsion. Evidence that lymphocytes contribute to rapid expulsion was demonstrated by adoptive transfer of thoracic duct $OX38^+$, $OX8^-$, $OX22^-$ T helper cells from *T. spiralis* infected rats to naïve rats (2). When these cells were transferred together with serum antibody protection was conferred on the recipients; however, protection was not optimal in this system, as only 40-60% reduction of the challenge dose was expelled. The cells transferred were collected from the thoracic duct three days after an oral infection. Therefore these cells are T-cells which were activated in the intestine and not T-cells which are activated later in the course of *T. spiralis* infection by muscle larvae. For complete worm rejection, it is likely that the potent antigenic stimulation provided by *T.spiralis* muscle larvae is essential. This is one explanation for the weak protection demonstrated in the thoracic duct cell transfer model. Thoracic duct T cells were also purified into two separate T-cell populations, $OX22^+$ and $OX22^-$ cells, and transferred to rats. Interestingly, $OX22^+$ cells induced intestinal mastocytosis but were not protective, while $OX22^-$ cells induced eosinophilia and were protective (121). A pivotal role in protection for eosinophils was not demonstrated, and it is not clear that the inflammatory changes observed were causal in protection. This adoptive transfer model does not reproduce a level of protection similar to

rapid expulsion. We speculate that T lymphocytes activated in the skeletal muscle are critical in promoting rapid expulsion.

D. The mucosal phenotype of rat bone marrow-derived mast cells (BMMC) and RBL-2H3 cells

We chose to use rat bone marrow-derived mast cells (BMMC) and RBL-2H3 cells to investigate whether parasite specific immune complexes could activate mast cells via Fc receptors. RBL-2H3 cells are a clonal cell line derived from a rat basophilic leukemia tumor. This cell line is the best characterized clonal model for in vitro studies of mucosal mast cells. Rat BMMC grown in culture with IL-3 and SCF display a superior homogenous mucosal phenotype when compared to other primary mast cell models such as peritoneal mast cells or mouse BMMC (73). Comparative studies of rat BMMC with its mucosal *in vivo* counterpart have demonstrated that these cells are biochemically and functionally similar (73). BMMC granules stain uniformly blue with Alcian blue, a dye that binds sulphated acid mucopolysaccharides and differentiates mucosal from connective tissue mast cell phenotypes in the rat (42). In addition, BMMC granules contain RMCP-II that is unique to mucosal mast cells (58). Cross-linking of surface bound IgE stimulates the release of RMCP-II, β-hexosaminidase, and leukotriene C_4 and TNF-α from BMMC (72, 73). The influence of IgG and immune complexes formed by IgG on BMMC has not been characterized previously. Mast cells are implicated in immunity to parasitic nematode infection, yet the mechanism for how they are activated to effect worm expulsion has remained elusive. One of the aims of this research was to investigate binding and activation of BMMC by IgG

containing immune complexes. We compared BMMC with the RBL-2H3 cell line, a thoroughly characterized model of the rat mucosal mast cell.

E. *Trichinella* antigens and IgG immune complexes

We utilized for our studies rat monoclonal IgGs (IgG1, IgG2a, IgG2b, and IgG2c) and polyclonal IgE that are specific for the immunodominant *N*-linked glycans produced exclusively by first-stage T*. spiralis* larvae. These antibodies bind to tyvelose, a 3, 6-dideoxy-D-arabinohexose that is terminally linked to tri- and tetra-antennary N-linked glycans (40). The glycans modify several glycoproteins synthesized by stichocytes and secreted by larvae as they enter the intestine. Excretory-secretory antigens are rich in tyvelose bearing glycoproteins. In addition, tyvelose modifies glycoproteins on the surface of larvae. Tyvelose is highly immunogenic and is bound by antibodies that mediate rapid expulsion in suckling rats. Interaction between *T. spiralis* antigens and antibodies are thought to inhibit the parasite within the intestinal environment by promoting mucus entrapment (30). Invasion of the intestinal epithelium by *T. spiralis* can be studied *in vitro* (74). Using this system, it was demonstrated that immune complexes form large aggregates and create cap-like structures on the anterior end of the parasite (78). This forms a physical barrier which interferes with sensory reception and impedes epithelial invasion. In addition, antibodies to tyvelose can encumber or immoblize larvae as they migrate through epithelial cells. Because of the structural nature of these immune complexes, we hypothesized that they would efficiently aggregate FcRs and cause activation of mast cells.

F. Fc receptors

Mast cell activation by Fc receptor engagement is regulated by cytoplasmic domains found in the receptor itself (α-chain) or in associated co-receptors (β and γ chains). Activating motifs (ITAMs) are inhibited by aggregation with receptors bearing inhibitory motifs (ITIMs) (97). The outcome of receptor engagement depends upon the properties of the specific receptors engaged and also on the quantity of receptor aggregation. High affinity Fc receptors bind to monomeric immunoglobulin or to immune complexes. Low affinity Fc receptors bind only complexes.

RBL-2H3 cells express the high affinity IgE receptor (FcεRI) and the low affinity, inhibitory IgG receptor (FcγRII) (27). While mRNA encoding FcγRIII is detectable in RBL-2H3 cells (44), surface expression is not evident (our results). There are at least 8 different isoforms (A-H) of this FcR identified in the rat, suggesting greater biologic FcγR plasticity in this species (44). Fc receptor expression in rat BMMC has not been described.

Human mast cells treated with IFN-γ express a high affinity Fc gamma receptor (FcγRI) that induces mast cell degranulation upon aggregation with IgG containing immune complexes (89). Aggregation of either FcεRI or FcγRI releases 22 different cytokines or chemokines (88). Interestingly, FcγRI aggregation released greater quantities of some of these inflammatory mediators when compared to FcεRI (88), demonstrating the potential of mast cells to be differentially activated by FcRs. It has not been determined whether rat mast cells can express FcγRI.

A novel FcR, FcγRIV, has recently been discovered on mouse myeloid cells (86). This receptor demonstrates distinct IgG isotype specificity for

IgG2a and IgG2b, thus providing another level of regulation inducible by IgG immune complexes. This FcR type has not yet been described in the rat.

The role of Fc receptor ratios (activating versus inhibitory) in modulating cellular responses that in turn influence immunity is only beginning to be appreciated (87). IgG immune complex binding and activation of rat mucosal mast cells via a Fc gamma receptor has not been documented. Because of the potential diversity of FcR on rat mast cells and the different antibody isotypes generated in parasitic infection, I postulated that immune complex interactions with FcR on mast cells are an important mechanism of regulation in parasitic infection.

G. Objectives of study

Rapid expulsion of *T. spiralis* from the rat occurs in conjunction with mast cell activation, and pharmacologic inhibition of 5-HT released from mast cells impairs expulsion (127). In addition rapid expulsion is antibody dependent. Thus, we desired to investigate a direct role for immune complex activation of the mast cell via FcR. We initially cultured mucosal mast cells from rat bone marrow in parallel with the mucosal mast cell line RBL-2H3 in order to pursue *in vitro* studies of immune complex binding and activation of mast cells. Because IgG2a binds to and triggers mast cell degranulation *in vitro*, we desired to further analyze the mechanism of action for this isotype in rapid expulsion *in vivo*. Our goals were to determine whether IgG2a-mediated protection was dependent on the Fc portion of the antibody or on complement. We diversified our approach to the question by using a parenteral immunization model to further our investigations on rapid expulsion.

Parenteral immunization is a very robust model of rapid expulsion and eliminates many of the confounding effects of intestinal inflammation induced by primary oral infection.

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CHAPTER TWO

Receptor binding and activation of mucosal mast cells by IgG complexed with nematode glycoproteins[*]

[*] Seana M. Thrasher, David Holowka, and Judith A. Appleton. Receptor binding and activation of mucosal mast cells by IgG complexed with nematode glycoproteins. Manuscript in preparation.

Abstract

Our aim is to test the role of mucosal mast cells in expulsion of *Trichinella spiralis* from the intestinal epithelium during a challenge infection. As part of this evaluation, we cultured mast cells from the bone marrow of rats for *in vitro* studies and compared them in a variety of assays with the model mucosal mast cell line, RBL-2H3. Rat bone marrow-derived mast cells (BMMC) displayed a homogenous mucosal phenotype as determined by uptake of Alcian blue and detection of rat mast cell protease-II (RMCP-II). BMMC were labeled with the mast cell specific ganglioside GD_{1b} antibody. In comparison, RBL-2H3 were less granular, produced less β-hexosaminidase, and contained little to no RMCP-II. We determined by flow cytometric analysis that immune-complexes formed with parasite glycoproteins and tyvelose-specific rat IgG1, IgG2a, and IgG2b but not IgG2c bound to Fc receptors on RBL-2H3 and BMMC. Blocking experiments revealed that IgG1 and IgG2a complexes bound to the high affinity IgE receptor (FcεRI) and a low affinity IgG receptor (FcγRII). IgE bound FcεRI and induced degranulation. We found that IgG2a but not IgG1 complexes induced degranulation of RBL-2H3; however, neither isotype induced significant degranulation of BMMC. IgG2b bound only FcγRIIb and did not induce degranulation. These results were confirmed with the highly characterized anti-DNP IgE and IgG2a, each complexed with DNP-BSA. BMMC had an increased expression of FcγRII and reduced expression of FcεRI compared to RBL-2H3 cells. Poor stimulation of BMMC by IgG complexes is postulated to be due to inhibition by FcγRII combined with a weak FcεRI signal. Our results suggest that rat mucosal mast cells are refractory to activation by IgG.

Introduction

Infection of rodents with the parasitic nematode *Trichinella spiralis* causes pronounced intestinal mastocytosis. Systemic release of mast cell mediators is coincident with parasite expulsion during primary and secondary infections (25, 34). Transgenic mice lacking the gene for mucosal mast cell specific, mouse mast cell protease-I (MMCP-I) demonstrate delayed expulsion of a primary infection with *T. spiralis* (19). In rats, mucosal mast cell specific rat mast cell protease-II (RMCP-II) is released during adult worm expulsion of *T. spiralis* (34) and during expulsion of secondary infections (25). Immunity to a challenge infection with *T. spiralis* has been characterized as intestinal anaphylaxis due to the rapid kinetics of worm expulsion concurrent with mast cell activation (17). This protective immunity, called rapid expulsion, occurs only in the rat and eliminates 99% of first-stage larvae from the intestine within hours (1). Rapid expulsion is antibody dependent but requires an unidentified cellular component (6). IgE as well as IgG isotypes (IgG1, IgG2a, and IgG2c) all confer protection (6). We hypothesized that these antibodies might form immune complexes which interact with Fc receptors on immune cells. Various mast cell mediators are elevated in serum and tissues during parasite expulsion; however, a role for antibodies in mast cell activation has not been documented.

We chose to use rat bone marrow-derived mast cells (BMMC) to investigate whether parasite specific immune complexes could activate mast cells via Fc receptors. Rat BMMC grown in culture with IL-3 and SCF display a superior homogenous mucosal phenotype when compared to other mast cell models such as RBL-2H3, peritoneal mast cells, or mouse BMMC (22). Comparative studies of rat BMMC with its mucosal *in vivo* counterpart have

demonstrated that these cells are biochemically and functionally similar (22). BMMC granules stain uniformly blue with Alcian blue, a dye that binds sulphated acid mucopolysaccharides and differentiates mucosal and connective tissue mast cell phenotypes in the rat (13). In addition, BMMC granules contain the mucosa-specific mast cell protease, RMCP-II (22).

Antibodies activate mast cells by binding and aggregating Fc receptors (FcR) on the cell surface. Rat mast cells transcribe FcεRI and FcγRII. These receptors are expressed on the surface of RBL-2H3 cells (9). FcεRI is the high affinity IgE receptor that triggers mast cell degranulation when aggregated with either IgE or IgG2a and antigen (7). FcγRIIb is a low affinity IgG receptor that, when aggregated with FcεRI, impedes mast cell degranulation (11). Activating FcγRII have been identified on mouse mast cells but not on rat mast cells (8). FcγRIII is a low affinity IgG receptor that activates mouse mast cells and induces degranulation (33). Transcripts of FcγRIII have been demonstrated in RBL-2H3 cells (14), but surface expression and function on rat mast cells has not been described. There are at least 8 different FcγRIII isoforms (A-H) identified in the rat, suggesting that greater Fc receptor plasticity occurs in this species (14). The significance of Fc receptor ratios (activating versus inhibitory) in modulating cellular responses is only beginning to be appreciated (27). FcγR-mediated activation of rat mucosal mast cells has not been documented.

In this report, we describe results of experiments performed with RBL-2H3 cells and BMMC. We confirmed that BMMC are mucosal in phenotype and are more typical of mucosal mast cells than RBL-2H3 cells. The cells were compared for binding and activation by immune complexes, and important differences were found, suggesting that rat mucosal mast cells may be

refractory to activation by IgG due to a low activating to inhibitory (A/I) FcR ratio (27).

Materials and Methods

Rats

Female Lewis strain rats (8 to 12 weeks old) were bred and maintained in the James A. Baker Institute for Animal Health vivarium. All rodents were housed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care.

Immunoglobulins

T. spiralis-specific monoclonal rat antibodies of four isotypes (IgG1, IgG2a, IgG2b, and IgG2c; clones 9D4, 18H1, 10G11, and 9E6) were previously characterized (2). Ascites fluid containing these monoclonal antibodies was purchased from Harlan (Indianapolis, IN), and heat inactivated at 56°C for 30 minutes before antibody precipitation with 40% saturated $(NH_4)_2SO_4$ as described previously (10). Monoclonal mouse anti-DNP IgE was purified as described (30), and rat anti-DNP IgG2a was purchased (American Research Products, Inc.; Belmont, MA). For preparation of polyclonal IgE, rats were infected orally with 2,000 *T. spiralis* first-stage larvae (L1) and re-infected 30 days later with the same dose. Rats were bled by cardiac puncture under isoflurane anesthesia 1 week after the second infection. Sera were stored at -80°C until purified by affinity chromatography using mouse anti-rat IgE antibodies (A2 and B5) as described by Bell et al.

(6). Antibodies were dialyzed against 0.85% normal saline and stored at -20°C.

Antigens

T. spiralis first-stage larvae (L1) were recovered from muscles of irradiated AO rats by digestion with 1% pepsin in acidified water (Crumm 1977). Rats had been infected at least 28 days prior to collection of larvae. Excretory-secretory antigen (ESA) was obtained from overnight cultures of L1 as described previously (2). Crude antigen (cAg) from L1 was prepared from whole worm homogenate as described (3), except that detergent was omitted from the protocol. BSA was conjugated with an average of 15 DNP groups (DNP-BSA, multivalent antigen) as described (30), dialyzed against PBS, and stored at 4°C. ESA was dialyzed and cAg was prepared in Dulbecco's PBS (DPBS) and stored at -20°C.

Cell culture

All cell culture materials were purchased from Gibco (Grand Island, NY), unless otherwise noted. Monolayer cultures of RBL-2H3 cells (5) were maintained in minimum essential medium (MEM) supplemented with 20% fetal bovine serum (Atlanta Biologicals, Inc.; Lawrenceville, GA) and 50 µg/mL gentamicin sulfate. Cells were grown at 37°C in 5% CO_2, and passaged by trypsinization weekly. For experiments, cells were collected 3-5 days after passage. BMMC were isolated and propagated using a protocol modified from Haig et al. (16). Briefly, Lewis rats were euthanized by CO_2 inhalation and cervical dislocation. Femur bones were dissected, and bone marrow flushed with sterile Hank's balanced salt solution (HBSS). Cells were washed five

times in HBSS then resuspended at 0.33-0.5 X 10^6 cells/mL, and cultured in Dulbecco's modification of Eagle's medium (DMEM) supplemented with 20% horse serum, 1 mM Hepes buffer, 4 mM L-glutamine, 100 U/mL penicillin, 100 µg/mL streptomycin, 50 µg/mL gentamicin, 100 ng/mL recombinant rat interleukin-3 (IL-3), and 50 ng/mL recombinant rat stem cell factor (SCF) (Peprotech; Rockyhill, NJ). Cells were maintained at 37°C in 5% CO_2, and resuspended (without trypsin) for passage every 3-5 days, when cell density reached 1 X 10^6 cells/mL. Experiments were conducted with cells harvested between 14-28 days of culture.

Cytology

Cytospins of BMMC were prepared at intervals by centrifuging aliquots of cells at 500 rpm for 5 minutes (Shandon Cytospin 2). Slides were air dried, stained with Alcian blue (Sigma; St. Louis, MO) for 10 minutes, and counter-stained with Safranin-O for 10 minutes (Sigma; St. Louis, MO). Coverslips were mounted with glycergel (DakoCytomation,Inc; Carpinteria, CA). A total of 100 cells were evaluated (stained/unstained) in high power fields (40X) of slides prepared from individual flasks. The percentage of cells stained blue was averaged and the mean value of three flasks reported. Slides were examined on an Olympus BX51 microscope and photographed with a DP-12 digital camera system (Melville, NY). Total cell counts were estimated from each of the three flasks using a hemacytometer (Reichert; Buffalo, NY).

RMCP-II detection in BMMC and RBL-2H3 cell lysates

Mouse anti-RMCP-II (21) was generously provided by Dr. Hugh Miller (University of Edinburgh). BMMC were sampled on days 11 and 28 in culture

and lysed using 1% Triton-X 100. RBL-2H3 cells were similarly prepared for comparison. Protein concentrations of lysates were measured with the Biorad protein assay kit (Biorad Laboratories Inc.; Hercules, CA). BMMC and RBL-2H3 lysates (20 µg and 30 µg per well) were resolved in a 10% discontinuous sodium dodecyl sulfate-polyacrylamide reducing gel (SDS-PAGE) at 25 mÅ for 1.25 hours. Gels were blotted onto nitrocellulose membranes at 400 mA for 1 hour, then blocked in DPBS supplemented with 7.5% non-fat dry milk (NFDM) and 0.2% Tween 20 at 4°C overnight. Membranes were incubated with antibody by gently rocking for 1 hour at room temperature. Mouse anti-RMCP-II was diluted (1:500) in DPBS supplemented with 2% NFDM and 0.2% Tween-20. The membrane was washed 5 times with DPBS and 0.2% Tween-20. Goat anti-rat IgG horseradish peroxidase conjugate (MP Biomedicals; Aurora, Oh), was diluted (1:1500) in DPBS supplemented with 2% NFDM, 0.2% Tween 20, and 10% normal goat serum. Membranes were incubated and washed 5 times as before. Antibody binding was detected with a chemiluminescent substrate (ECL reagent; Amersham Pharmacia Biotech; Piscataway, NJ) and autoradiography with Classic Blue Sensitive x-ray film (Laboratory Products Sales; Rochester, NY). Film was developed in a Kodak X-OMAT 1000 A processor (Rochester, NY) and scanned. The image was prepared using Adobe Photoshop and Microsoft Powerpoint.

Flow cytometric analysis of immune complex and antigen binding

Rat monoclonal IgG1, IgG2a, IgG2b, IgG2c (2), and polyclonal IgE (6), were conjugated with Alexa Fluor-488 (Molecular Probes; Eugene, OR). Briefly, immunoglobulins were dialyzed in PBS (pH 8.5) overnight at 4°C. Alexa Fluor-488 (100 µg/mL) was added to immunoglobulin (1.0 mg/mL) and

incubated at room temperature for 4 hours or overnight. Samples were dialyzed in PBS overnight and stored with 0.1% sodium azide at 4°C in a light-protected container until use. Molar dye:protein ratios were estimated to be between 3 – 13.

BMMC and RBL-2H3 cells were washed in PBS three times then resuspended in PBS at 0.5 X 10^6 cells/mL. Cells were incubated with conjugated immunoglobulins (10 µg/mL) for 15 minutes and then ESA or cAg (10 µg/mL) was added. Cells were incubated on ice for 1 hour then washed three times with PBS and fixed in 2% paraformaldehyde. In some experiments, Fc receptors were blocked with mouse anti-rat FcεRI or mouse anti-rat FcγRII (BD Pharmingen; San Diego, CA). Blocking antibodies were added to cells separately or together 30 minutes prior to immune complex addition. Immune complex binding was detected with a FACSCalibur flow cytometer (BD Biosciences; San Jose, CA) and data were analyzed using Cell Quest or Flow Jo software. Figures were generated using Flow Jo and Microsoft Powerpoint.

Direct binding of ESA and cAg to cells was tested by incubating cells for 1 hour on ice with either ESA or cAg (10 µg/mL). We found that rat IgG2c immune complexes did not bind to mast cells, so rat IgG2c-Alexa 488 (10 µg/mL) was used to detect direct antigen binding to cells. Unbound antigen was removed from cells by washing with PBS and the cells were incubated with IgG2c for 30 minutes, washed 3 times with PBS, fixed in 2% paraformaldehyde, and analyzed by flow cytometry.

β-Hexosaminidase assay

Degranulation was assessed by a modified colorimetric assay that measures β-hexosaminidase release from cells (32). Briefly, cells were cultured overnight in either 48 well (2.50 X 10^5 cells per well) or 96 well (1.25 X 10^5 cells per well) plates. Both BMMC and RBL-2H3 cells were adherent. Cells were washed twice with Tyrodes buffer (20 nM HEPES, 135 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% BSA, and 1.8 mM $CaCl_2$ (pH 7.4)). Immunoglobulins were diluted in Tyrodes buffer, added to cells 15 minutes prior to antigen. Cells were incubated with complexes for 1 hour at 37°C. Calcium ionophores (1 μM), ionomycin (Sigma; St. Louis, MO) or A23187 (Sigma; St. Louis, MO), in the presence of phorbol ester-12 myristate-13 acetate (PMA) (50 nM) (Sigma; St. Louis, MO) served as positive controls for cell stimulation. For 48 well plates, 50 μl culture supernatant were incubated with 200 μl of 1 mM *p*-nitrophenyl *N*-acetyl-β-D-glucosaminide (Sigma; St. Louis, MO) dissolved in 0.05 M citrate buffer (pH 4.5) for 1 hour at 37°C. The reaction was stopped with 500 μL of 0.1 M sodium carbonate buffer. Substrate was measured at 400-405 nm using either an Ultraspec 2100 pro spectrophotometer (Amersham Pharmacia Biotech; Piscataway, NY) or an ELISA plate reader (Bio-Tek Instruments; Winooski, VT). β-hexosaminidase release was reported as the percent of total enzyme per well. Samples were run in triplicate and the means reported. Total β-hexosaminidase concentration was determined by extracting (1:1) the remaining buffer and cells with 1% Triton X-100 (Sigma; St. Louis, MO). A 50 μl aliquot was removed and analyzed as described.

Statistical analysis

Data were evaluated by analysis of variance (ANOVA) and Scheffe's comparison of means using Statistix (Analytical Software; Tallahassee, FL). Differences were considered statistically significant when $p < 0.05$.

Results

Culture and phenotyping of BMMC.

Bone marrow cultures were passaged every three days for fifteen days. Cytospin preparations were made from aliquots collected at the time of passage. Numerous, large cytoplasmic granules were detected with Alcian blue, indicating that BMMC developed a homogenous mucosal phenotype (Figure 2.1 A). When compared to RBL-2H3 cells (Figure 2.1 B), BMMC had larger, more distinct granules that stained with greater intensity. Bone marrow cultures contained 95% mucosal mast cells by 12 days in culture and 99% mucosal mast cells by 15 days (Figure 2.1 C). Cell growth and viability diminished after 30 days in culture.

RMCP-II is an enzyme found only in mucosal mast cells (15). Cell lysates prepared from bone marrow cells at day 11 and 28 in culture were compared with lysates of RBL-2H3 cells in Western blots developed with antibody to RMCP-II. A 24 kDa protein was readily detected in bone marrow cell lysates; however, similar protein loading of RBL-2H3 cell lysate was not sufficient for RMCP-II detection (Figure 2.1 D). In addition, when equivalent numbers of BMMC and RBL-2H3 cells were assayed for β-hexosaminidase, cells in bone marrow cultures (14 days of culture or later) yielded twice the quantity of β-hexosaminidase when compared to RBL-2H3 cells (Figure 2.1

E). We confirmed that ganglioside-specific monoclonal antibody AA4 bound to BMMC. AA4 detects two derivatives of ganglioside GD1b that are unique to mast cells (28) and present on all rat derived mast cells including BMMC (18). Surface expression of GD_{1b} was approximately 3-fold greater on RBL-2H3 cells than BMMC (Figure 2.1 F & G). Thus we documented that cells cultured from bone marrow in the presence of SCF and IL-3 were uniformly of a mucosal mast cell phenotype. In subsequent experiments, we assayed cells harvested from cultures between 14 and 28 days and refer to these cells as BMMC.

Specificity of immune complexes for FcR on RBL-2H3 cells and BMMC. Because IgG as well as IgE are protective against *T. spiralis in vivo* (6) and mast cells are implicated in immunity, we sought to ascertain whether IgG immune complexes could activate mast cells via Fc receptors. We found that immune complexes containing IgG1, IgG2a, and IgG2b bound to both BMMC and RBL-2H3 cells (Figure 2.2 A) while IgG2c did not (data not shown). By blocking with specific antibodies for receptors, we found that IgG1-ESA and IgG2a-ESA bound to FcεRI and FcγRII on both cell types (Figure 2.2 A), while IgG2b-ESA bound only FcγRII (Figure 2.2 A). IgE-ESA bound to FcεRI. Figure 2.2 B summarizes the geometric mean fluorescence intensities of RBL-2H3 cells and BMMC bound by immune complexes and the reduction in GMFI obtained when complexes were competed with receptor-specific antibodies. Substitution of *T. spiralis* crude antigen (cAg) for ESA did not alter the outcome of the experiment (data not shown). Immune complex binding was similar for FcRs on RBL-2H3 cells and BMMC despite the greater heterogeneity of receptor expression by BMMC.

Figure 2.1: Comparison of properties of RBL-2H3 cells with BMMC. Cytospins prepared from BMMC at 21 days in culture (A) and RBL-2H3 cells (B) were stained with Alcian blue/Safranin-O. (C) Growth and staining properties of bone marrow cells cultured in the presence of IL-3 and SCF. Cells containing granules detected with Alcian blue were classified as mast cells. Values represent the mean +/- 1 SD (n = 3). (D) Detection of RMCP-II in Western blots of BMMC and RBL-2H3 cell lysates. Cells were collected from bone marrow cultures after 11 or 28 days. (E) β-hexosaminidase content was quantified in cell lysates prepared from cells after 21 days in culture and is reported as optical density (OD) at 400 nm per 1.25 X 10^5 cells. Values represent the mean +/- 1 SD (n = 12 wells). Detection of rat mast cell specific ganglioside, GD1b, with FITC-conjugated antibody by flow cytometric analysis on (F) RBL-2H3 cells and (G) BMMC. Unstained cells (shaded grey) and nonspecific IgG-FITC (grey line) served as negative controls.

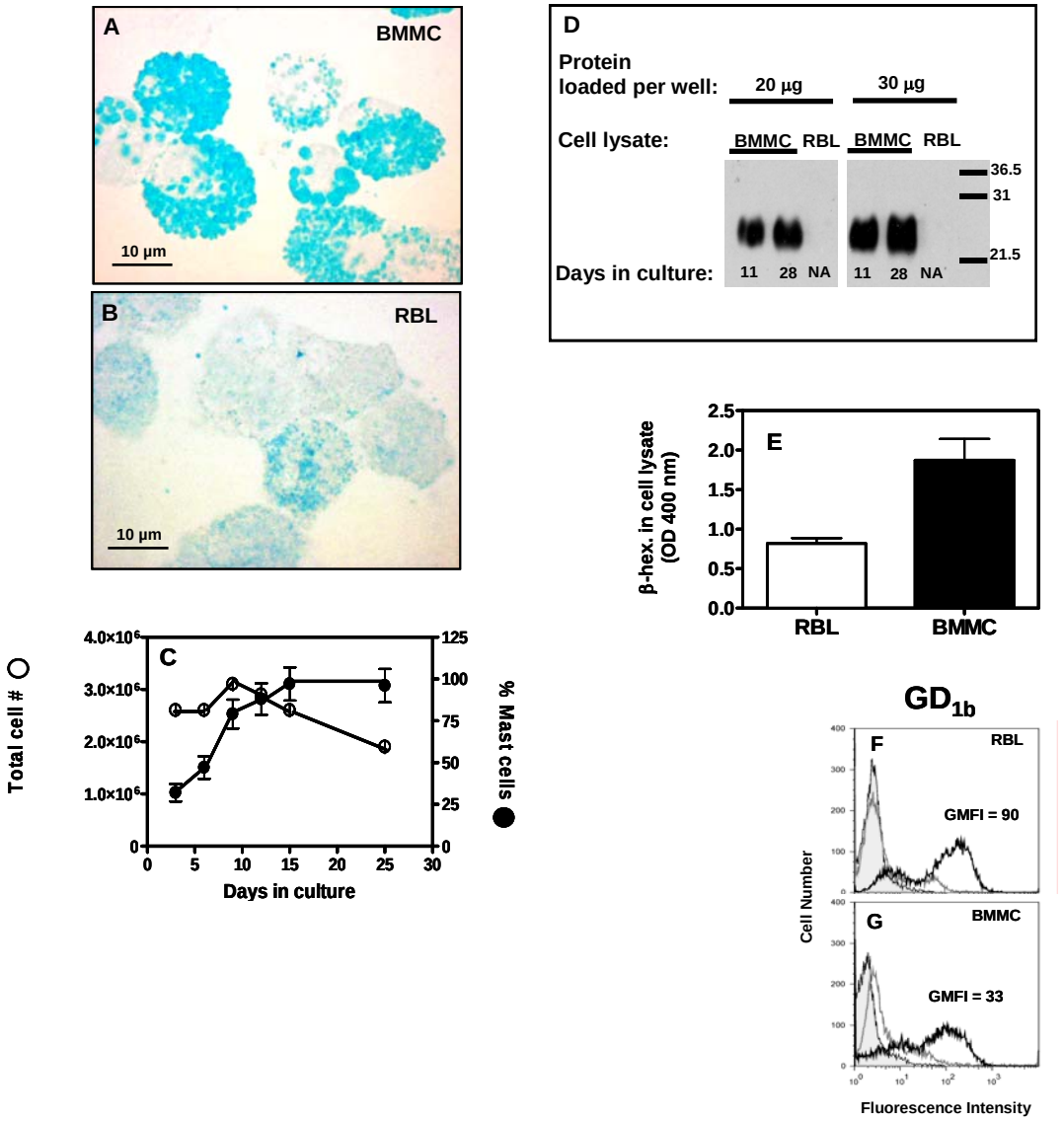
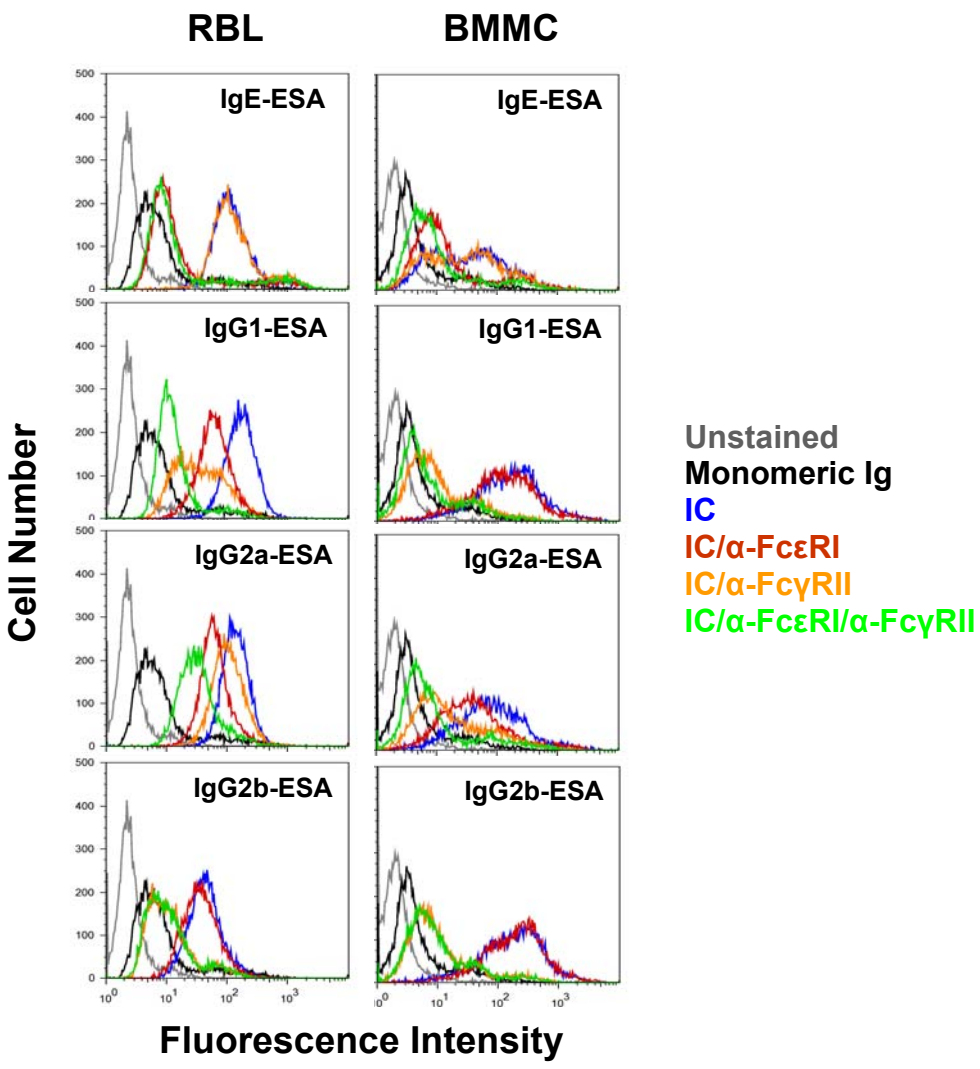

A
BMMC
10 µm
B
RBL
10 µm
C
Total cell # ○
% Mast cells ●
Days in culture
D
Protein loaded per well:
20 µg
30 µg
Cell lysate:
BMMC RBL BMMC RBL
36.5
31
21.5
Days in culture:
11 28 NA 11 28 NA
E
β-hex. in cell lysate (OD 400 nm)
RBL
BMMC
GD1b
F
RBL
GMFI = 90
G
BMMC
GMFI = 33
Cell Number
Fluorescence Intensity

Figure 2.2: Flow cytometric analysis of binding by immune complexes to Fc receptors on RBL-2H3 cells and rat BMMC. (A) Fluorescence intensity of cells treated with immune complexes. Unstained cells (grey line) and monomeric IgG2c-Alexa 488 (black line) served as negative controls. Binding was blocked with antibodies specific for FcεRI (red line) or FcγRII (orange line) separately or in combination (green line). Dye: protein molar ratio for antibodies ranged from 3 to 13. (B) Reduction in geometric mean fluorescence intensity of immune complex (IC) binding to RBL-2H3 cells or rat BMMC in the presence of receptor blocking antibodies.

A



B

% Reduction in GMFI

BMMC	IC GMFI	α-FcεRI	α-FcγRII	α-FcεRI/ α-FcγRII
IgE-ESA	30	57	10	70
IgG1-ESA	138	25	93	95
IgG2a-ESA	72	49	76	89
IgG2b-ESA	155	0	94	94
RBL				
IgE-ESA	125	87	0	86
IgG1-ESA	163	63	79	91
IgG2a-ESA	134	54	24	75
IgG2b-ESA	43	16	72	74

Surface expression of the inhibitory receptor FcγRII is predicted to affect mast cell activation by IgG2a and IgG1 immune complexes. We found that IgG2b-Alexa 488 complexed with *T. spiralis* ESA bound exclusively to the inhibitory receptor on BMMC and RBL-2H3 cells. As determined by flow cytometric analysis, BMMC (Figure 2.3 B) had a 4-fold greater surface expression of FcγRII compared with RBL-2H3 cells (Figure 2.3 A). Additionally, IgE-ESA immune complexes bind exclusively to FcεRI. RBL-2H3 cells (Figure 2.3 C) had a 4-fold greater surface expression of FcεRI than BMMC (Figure 2.3 D). Thus, we find that BMMC have a lower A/I ratio than RBL-2H3 cells.

Activation of RBL-2H3 cells and BMMC by immune complexes.

The experiments described above showed that three isotypes of IgG were bound by RBL-2H3 cells and BMMC. Next we assayed those immune complexes for mast cell activation. It has been reported the IgG2a complexes bind and activate mast cell degranulation via FcεRI (7). We titrated parasite specific IgE (0.15 to 2.5 μg/mL) and IgG2a (0.15 to 2.5 μg/mL) with ESA (1 μg/mL). Both isotypes caused RBL-2H3 degranulation (Figure 2.4 A). Optimal immunoglobulin concentration for degranulation was 2.5 μg/mL. By blocking FcεRI with monomeric IgE IR 162, we confirmed that mast cell activation by IgG2a was mediated by FcεRI aggregation (Figure 2.4 B). Although IgG1 bound the FcεRI and FcγRII in a manner identical to IgG2a, it failed to stimulate significant degranulation in RBL-2H3 cells or BMMC above spontaneous β-hexosaminidase release (Figure 2.4 C). IgG2b did not induce degranulation, a result that is compatible with its binding solely to the inhibitory receptor, FcγRII (Figure 2.4 C).

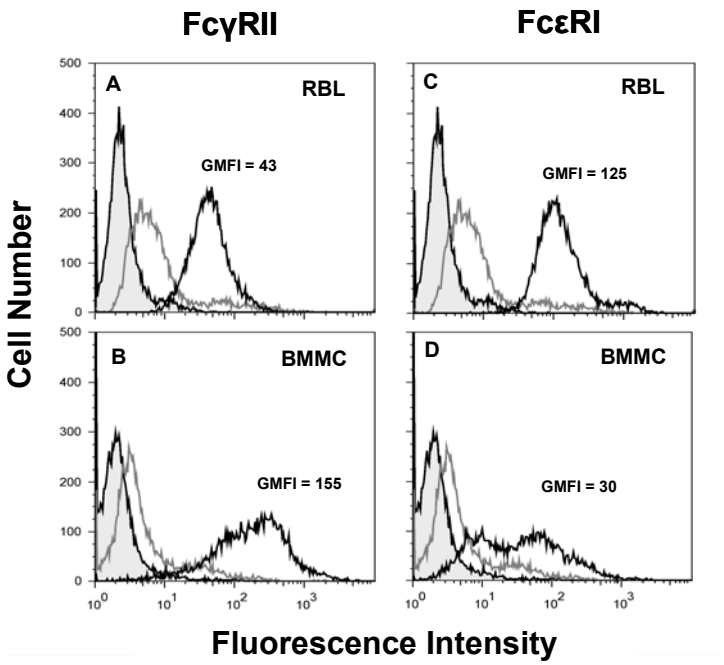
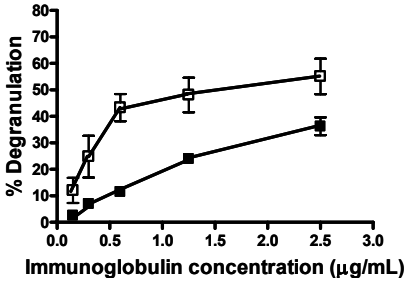
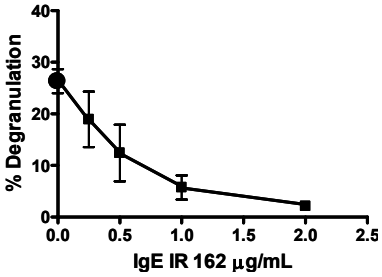


Figure 2.3: Comparison of surface expression of FcγRII and FcεRI on RBL-2H3 (A, C) cells and BMMC (B, D). Cells were labeled with Alexa-488 conjugated IgG2b or IgE (10 μg/mL) complexed with ESA (10 μg/mL) and analyzed by flow cytometry. Immune complex binding is represented with a black line, conjugated monomeric IgG2c is represented by a grey line, and unstained cells are shaded. Experiments were repeated at least three times.

A



B



C

Immune complexes	RBL-2H3 Degranulation (%)	BMMC Degranulation (%)
IgG1-ESA	2.4	7.6
IgG2b-ESA	1.9	6.1
ESA	1.3	5.3
PMA/Ionomycin (50 nM/1µM)	61	38

Figure 2.4: Degranulation of RBL-2H3 cells induced by antibodies complexed with *T. spiralis* ESA. (A) Immune complexes formed by 1 µg/mL of ESA with increasing concentrations of IgE (□) or IgG2a (■) were incubated with RBL-2H3 cells. (B) Inhibition of IgG-induced degranulation by monoclonal IgE IR 162. Cells were incubated with various concentrations of IgE prior to adding complexes formed by anti-tyvelose IgG2a (2 µg/mL) and ESA (1 µg/mL). Symbols represent the mean +/- 1 SD of triplicate samples. (C) RBL-2H3 cells and BMMC were stimulated with IgG1 or IgG2b (2.5 µg/mL) and ESA (1.0 µg/mL). Values represent the average of two duplicates. Data shown are representative of three experiments.

We compared RBL-2H3 cell and BMMC activation by anti-tyvelose IgE and IgG2a in complex with ESA or cAg. In addition, we tested anti-DNP IgE and IgG2a in complex with DNP-BSA. We compared two different parasite antigen preparations in addition to DNP-BSA to determine whether differences in immune complex structure might enhance FcR aggregation and mast cell activation. IgE complexed with ESA, cAg, and DNP-BSA stimulated significant cell degranulation in both RBL-2H3 (Figure 2.5 A-C) and BMMC (Figure 2.5 D-F). IgG2a-ESA and IgG2a-DNP-BSA induced significant degranulation in RBL-2H3 cells (Figure 2.5 A, C); however, degranulation induced in BMMC was weak but reproducible, yet not statistically significant (Figure 2.5 D, F). IgG2a-cAg also did not induce significant degranulation of either RBL-2H3 cells (Figure 2.5 B) or BMMC (Figure 2.5 E). Treatment of RBL-2H3 cells with PMA or cytochalasin D prior to stimulation with immune complexes will enhance degranulation. We also stimulated BMMC with immune complexes in the presence of PMA or cytochalasin D in an attempt to enhance degranulation; however, neither compound improved degranulation of BMMC by IgG2a complexes (data not shown). Comparison of RBL-2H3 and BMMC responses to 50 nM PMA and 1 μM calcium ionophore A23187 confirmed that BMMC responded as well as RBL-2H3 cells to stimulation by these compounds (Figure 2.5 D-F). Thus, while BMMC are capable of degranulation responses to IgE or ionophores, they are refractory to IgG immune complexes.

Binding of parasite antigens to BMMC and effect on activation.

In order to determine whether the poor degranulation response by BMMC was due to a direct effect of parasite antigens on the cells, we assayed binding of ESA and cAg to the surfaces of BMMC and RBL-2H3 cells. ESA

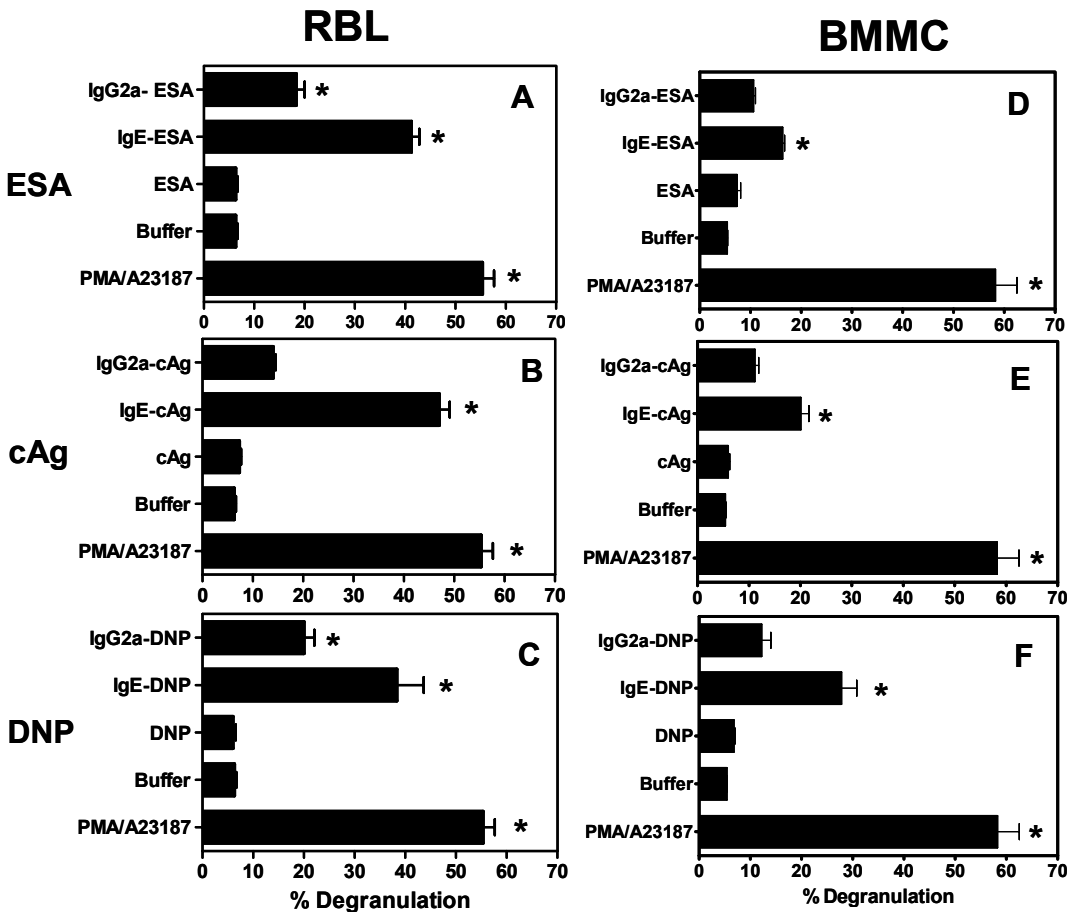


Figure 2.5: Degranulation responses of RBL-2H3 cells and BMMC. RBL-2H3 (A-C) and BMMC (D-F) stimulation with immune complexes formed with IgG2a or IgE specific for tyvelose (found in ESA and cAg), or DNP-BSA. Antibodies (10 µg/mL) were complexed with antigens (1 µg/mL), except for RBL-2H3 stimulation with anti-DNP-BSA IgE and IgG2a (2 µg/mL). Asterisks denote values that differed significantly from the buffer control ($p < 0.05$). Values graphed are means +/- 1 SD of three duplicates.

did not bind, while cAg bound to both cell types (Figure 2.6 A). To determine whether binding influenced responsiveness of the cells, we incubated cells with parasite antigen before stimulation with IgE-DNP-BSA. Treatment with ESA or cAg (10 μg/mL) before addition of IgE-DNP had no effect on BMMC degranulation (Figure 2.6 B).

Discussion

Rat BMMC grown in the presence of IL-3 and SCF have a homogenous mucosal phenotype characterized by prominent granules that stain with Alcian blue and by RMCP-II production (16). IgE cross-linking stimulates the release of RMCP-II, β-hexosaminidase, and leukotriene C_4 from BMMC (21). Rat BMMC also store TNF-α and upon IgE mediated stimulation release this cytokine (22). Studies of these cells have demonstrated that they are functionally and biochemically very similar to their *in vivo* mucosal counterpart (22). Because of this we chose to culture rat BMMC for our investigations concerning IgG mediated activation of mucosal mast cells. We compared BMMC to RBL-2H3 cells for mucosal properties and found that, compared with RBL-2H3 cells, BMMC contained larger cytoplasmic granules that uniformly stained with Alcian blue, as well as greater quantities of RMCP-II and β-hexosaminidase. Degranulation in response to stimulation with PMA and the calcium ionophore A23187 was equivalent for BMMC and RBL-2H3 indicating that BMMC were capable of degranulation after 14 days of culture *in vitro*.

Mast cells are implicated in immunity to *T. spiralis* infection, yet the mechanism by which they affect worm expulsion has remained undefined. We

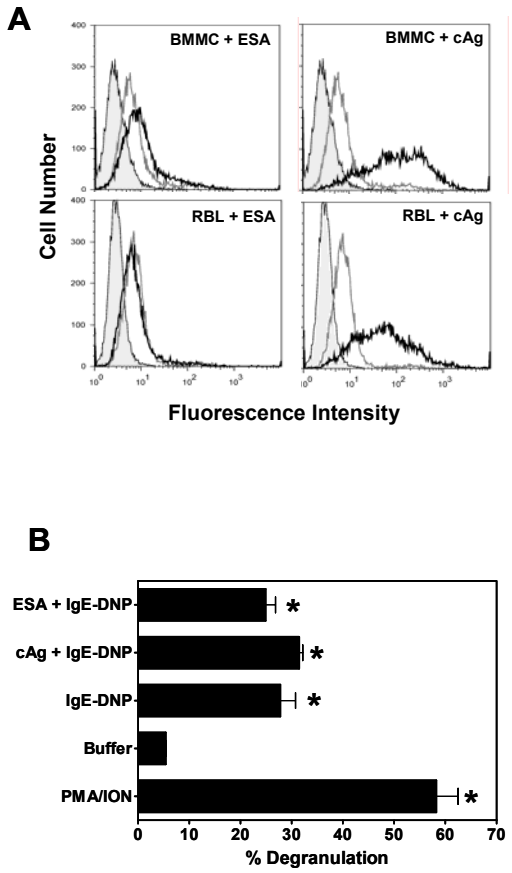


Figure 2.6: Direct antigen binding to BMMC and RBL-2H3 cells. (A) Flow cytometric evaluation of cells after incubation with antigens. ESA and cAg (10 µg/mL; black line) were incubated with BMMC and RBL-2H3 cells before addition of IgG2c-Alexa 488 (10 µg/mL). Unstained cells are represented by the shaded peak and cells incubated with conjugate alone are indicated by grey line. (B) Incubation with ESA or cAg (10 µg/mL) before addition of IgE (10 µg/mL) and DNP (1 µg/mL) did not significantly alter cell stimulation. Values are reported as the mean +/- 1 SD (n = 3). Asterisk denotes significant differences from cells treated with buffer ($p < 0.05$).

hypothesized that parasite-specific IgG immune complexes might activate mast cells via FcRs. Three IgG isotypes (IgG1, IgG2a, and IgG2c) are protective *in vivo* against a challenge infection with *Trichinella* (6). This protection is not mediated by antibodies alone, but requires cooperation with some yet unidentified cell or co-factor. Since mastocytosis is a prominent inflammatory feature of intestinal infection by *T. spiralis*, it is possible that immune complexes formed by IgG might interact with mast cell Fc receptors in promoting expulsion. Protective monoclonal antibodies are all specific for tyvelose, a sugar that modifies the termini of tri- and tetra-antennary glycans on *T. spiralis* glycoproteins (12). Tyvelose-specific antibodies have been shown to form large complexes with secreted products of *T. spiralis in vitro* (23), and are likely to be highly effective in aggregating FcRs on cell surfaces.

We found that IgG1, IgG2a, and IgG2b bound to Fc receptors on BMMC and RBL-2H3 cells. IgG2b, which is not protective *in vivo,* bound only to the FcγRII. Consistent with the inhibitory function of this receptor, IgG2b complexes did not stimulate degranulation. IgE bound only to FcεRI and caused degranulation in BMMC and RBL-2H3 cells. IgG2a and IgG1 bound to FcεRI and FcγRII on both cell types; however, only IgG2a elicited degranulation on RBL-2H3 cells via the FcεRI. IgG1 and IgG2a are very similar in structure and function and it is unclear why these two isotypes differ in their ability to activate mast cells. Rat IgG1 has been reported to stimulate degranulation in peritoneal mast cells; however, these experiments were preformed with polyclonal IgG that was purified with polyclonal reagents (24). Other evidence indicates that IgG1 can function as a blocking antibody that inhibits IgE mediated degranulation of RBL-2H3 cells (29). The functional discrepancies between IgG1 and IgG2a may be due to differences in binding

affinities for activating versus inhibitory FcRs. Alternatively, there may be an activating FcγRII or FcγRIII that binds IgG2a exclusively that has not been described in rat mast cells and that our reagents fail to distinguish.

Surface expression of the inhibitory FcγRII was greater while FcεRI surface expression was reduced on BMMC compared with RBL-2H3 cells. There is convincing evidence that FcγRII is not functional on RBL-2H3 cells (9) and our data support this conclusion. We found that cross-linking the FcεRI and FcγRII with anti-tyvelose IgG2b and IgE did not inhibit IgE-mediated degranulation of RBL-2H3 cells (data not shown). This provides a rational explanation for the greater response to IgG2a complexes of RBL-2H3 cells compared to BMMC. Alternatively, structural properties of parasite antigen-antibody complexes might be the reason for decreased responsiveness to IgG in BMMC. To address this, we assayed immune complexes formed with other antigens. We compared IgE and IgG2a complexed with DNP-BSA, ESA, and cAg. IgE-DNP-BSA consistently stimulated the greatest degranulation response in BMMC. IgE-ESA and IgE-cAg were active but induced weaker responses. This suggests that structural differences among the antigens may influence receptor aggregation and mast cell stimulation. Immune complexes formed with IgG2a and DNP, ESA, or cAg did not induce substantial degranulation in BMMC. Differences between BMMC stimulation induced by IgE and IgG2a is likely attributable to BMMC expressing a lower activating to inhibitory (A/I) FcR ratio than RBL-2H3, as a result of greater inhibitory FcγRII expression and lower FcεRI expression on BMMC. Future studies utilizing treatment with inflammatory cytokines to decrease expression levels of this receptor or RNA interference to prevent its surface expression would be informative for clarifying the significance of IgG in mast cell activation.

While it is known that mast cells express Toll-like receptors (TLRs) that promote or induce cytokine release (20, 31), a TLR specific for nematode parasite antigens has yet to be identified. *T. spiralis* TSL-1 antigens trigger significant cytokine and histamine release from a hybrid rat mast cell line (HRMC) (4, 26) in the absence of immunoglobulin. TSL-1 antigens are affinity purified from cAg by binding to tyvelose-specific antibody. They are distinct in composition from ESA that contains only secreted products not all of which bear tyvelose. The receptor that mediates activation by TSL-1 antigens has not been identified, nor has the physiologic significance of activation been determined. In our study, we investigated other related *T. spiralis* antigens for binding to and activation of mucosal mast cells. *T. spiralis* ESA did not bind to the surfaces of cells; however, cAg did bind to both BMMC and RBL-2H3 cells. We found that binding by cAg did not induce β-hexosaminidase release, nor did it inhibit degranulation induced by immune complexes. Thus parasite antigens neither promoted nor inhibited mucosal mast cell activation. Although it is possible that aggregation of parasite specific immunoglobulins with surface bound cAg has qualitative effects on mast cell mediator release, β-hexosaminidase release was similar with immune complexes containing ESA and cAg. This supports the conclusion that cAg did not alter responsiveness of cells to immune complexes.

Mucosal mast cells in the Th2 cytokine-rich environment induced by parasitic infection may have Fc receptor diversity that differs from that displayed by BMMC. The ability of the mast cell to change phenotype, to regulate Fc receptor diversity and isotype interactions, and to release a variety of inflammatory mediators merits further investigation in the context of parasitic infection. In this report, we have demonstrated that BMMC cultured

in vitro are a useful model for pursuing investigations of FcR driven activation because of their similarity to the mucosal mast cell *in situ*. Isotype specificity of FcRs were the similar for both RBL-2H3 cells and BMMC; however, BMMC had a lower A/I receptor ratio. The lower A/I ratio, rather than structural features of parasite antigen-antibody complexes, resulted in sub-optimal FcR mediated degranulation. Our data indicate that due to elevated expression of FcγRII, mucosal mast cells may be refractory to stimulation with IgG but not IgE.

Acknowledgements

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CHAPTER THREE

Rapid expulsion of *Trichinella spiralis* by tyvelose-specific rat IgG2a in a model of intestinal immunity[*]

[*] Seana M. Thrasher and Judith A. Appleton. Rapid expulsion of *Trichinella spiralis* by tyvelose-specific rat IgG2a in a model of intestinal immunity. Manuscript in preparation.

Abstract

Rats infected with the parasitic nematode, *Trichinella spiralis*, protect themselves against re-infection by a dramatic immune mechanism called rapid expulsion. Our aim is to clarify the mechanism of IgG2a-mediated expulsion of *T. spiralis* from the intestine during challenge infection. We hypothesized that IgG2a immune complexes interact with Fc receptors on leukocytes to promote the rapid expulsion of larvae from the intestine. Although correlative evidence suggests that expulsion involves mast cells, the mechanism of expulsion has not been elucidated. In previous studies, passive immunization of adult rats with parasite-specific monoclonal IgG2a caused expulsion of *T. spiralis* larvae within 24 hours (4). This protective mechanism occurred only when rats had been previously infected with an unrelated nematode, *Heligmosomoides polygyrus*. Infection with *H. polygyrus* likely activates innate immunity that cooperates with antibodies to cause expulsion. We demonstrate here that rapid expulsion occurs in Lewis rats infected with *H. polygyrus* and passively immunized with specific IgG2a. This immunity was associated with increased eosinophilia, mastocytosis, and mast cell activation. These changes were more dramatic in Lewis than in AO strain rats and correlated with better protection in Lewis rats. Mucus entrapment of larvae is a hallmark of rapid expulsion, but was not evident in IgG2a mediated expulsion. IgG2a mediated protection was Fc-dependent and did not require complement. Our data support an indirect influence of IgG2a in rapid expulsion that likely involves FcR-bearing cells.

Introduction

Rats previously infected with the parasitic nematode, *T. spiralis*, demonstrate a potent immune defense against secondary intestinal infection that has been called rapid expulsion (14). Within a few hours, 99% of first-stage larvae are expelled from the intestine (6). Mucus entrapment of larvae is a hallmark of rapid expulsion (9),(4) and has been shown to be mediated by antibodies that are specific for tyvelose, a sugar that is unique to the first-stage larva. Antibodies specific for tyvelose cause expulsion of larvae from the intestines of passively immunized neonatal rats.

During primary infection, first-stage larvae in muscles induce a substantial tyvelose-specific antibody response. The intestinal phase of infection induces goblet cell hyperplasia, mastocytosis, and eosinophilia in the small intestine (4). Mechanistic studies in adult rats have shown that antibody is necessary but not sufficient for expulsion, and that some cellular component or co-factor of the immune system is required (5). Rapid expulsion can be reproduced in adult rats by infection with a heterologous nematode, such as *Heligmosomoides polygyrus*, and passive transfer of *Trichinella* specific monoclonal antibodies (4). IgG1, IgG2a, and IgG2c are all protective in this model but IgG2b is not. IgG1 and IgG2c are protective in suckling rats (2) and these isotypes cause mucus entrapment of the parasite (8). However, IgG2a, like IgE, is not protective in neonatal rats and does not entrap larvae in mucus (8). In contrast, IgG2a and IgE are protective in adult rats infected with *H. polygyrus* (4), suggesting that antibodies cooperate with an innate cellular component to effect immunity. It is thought that the mechanism for IgG2a

mediated protection is different from the other IgG isotypes but similar to that of IgE (4).

In experiments reported here, we investigated further the mechanism of IgG2a-mediated protective immunity. We found that *H. polygyrus* infection alone caused limited but reproducible expulsion of *T. spiralis*, and this was associated with mast cell activation and other inflammatory changes in the intestine. IgG2a was protective against *T. spiralis* in rats infected with *H. polygyrus*, although mucus entrapment of larvae was not prominent; however, the magnitude of protection approached that of rapid expulsion demonstrated by naturally infected rats. Finally, IgG2a mediated protection in an Fc-dependent but complement independent, manner.

Materials and Methods

Rats and mice

Albino Oxford (AO), Lewis, PVG, LouM, and Brown Norway (BM) strain rats (8 to 12 weeks old) were bred and maintained in the James A. Baker Institute for Animal Health vivarium. Eight week old C57BL/10SgAiTac (WT) or C57BL/10ScNHsd (WT) mice were purchased from Taconic Animal Models (Germantown, NY) or Harlan (Indianapolis, IN), respectively. All rodents were housed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care.

Antibodies

Monoclonal rat anti-tyvelose IgG2a (clone 18H1.1) has been characterized (Appleton et al., 1988). 18H1.1 ascites was purchased from

Harlan (Indianapolis, IN), and normal rat serum (NRS) was collected by cardiac puncture from uninfected rats under isoflurane anesthesia. Sera or ascites were heat inactivated at 56°C for 30 minutes before antibody precipitation with 40% saturated $(NH_4)_2SO_4$ as described previously (9). Mouse anti-rat kappa chain (Mark-1) was precipitated with 40% $(NH_4)_2SO_4$ from ascites as described and biotinylated with N-hydroxysuccinimidobiotin (Sigma; Saint Louis, MO) in bicarbonate buffer (pH 9.5; 120 µg biotin per mg of antibody) for 4 hrs at room temperature. Horseradish peroxidase (HRP) - conjugated goat anti-rat IgG (H&L) was purchased (MP Biomedicals; Aurora, Oh).

Preparation of Fab'2

Fab'2 fragments were prepared from IgG2a according to a protocol modified from Rousseaux et al. (17). Briefly, IgG2a was dialyzed in 0.1M Na formate (pH 2.8) for 16 hours, dialyzed in 0.1M Na acetate (pH 4.2) for three hours, and digested with 2% (w/w) pepsin at 37°C for four hours. The reaction was stopped by dialysis in 0.1M Na acetate (pH 8.2) overnight at 4°C. Cleavage was confirmed by resolving fragments in 8% SDS PAGE non-reducing gels, blotting to nitrocellulose, and developing blots with goat anti-rat IgG-HRP. Binding efficiency for parasite specific IgG2a and IgG2a Fab'2 fragments was measured by ELISA. Briefly, polyvinyl 96-well plates were coated with 5 µg/mL *T. spiralis* crude antigen diluted in 10% Dulbecco's PBS (DPBS) overnight at 4° C. Plates were blocked with DPBS containing 7% non-fat dry milk (NFM). Serial dilutions of IgG2a and IgG2a Fab'2 fragments in DPBS containing 2% NFM were incubated for 1 hour at room temperature. Biotinylated Mark-1 (2 µg/mL) diluted in DPBS was incubated for 1 hour at

room temperature followed by 1:1,000 dilution of HRP conjugated-strepavidin in DPBS (BD Pharmingen, San Diego, CA). The plates were developed for 5 minutes with tetramethylbenzidine (TMB) (KPL Laboratories, Gaithersburg, MD) and stopped with 1 M H_3PO_4. Absorbance (450 nm) was measured with an ELISA plate reader (Bio-Tek Instruments, Winooski, VT). Antibodies and Fab'2 fragments were dialyzed in 0.85% saline and stored at -20° C until use.

Heligmosomoides polygyrus *and* Trichinella spiralis

H. polygyrus was propagated in C57BL mice following methods described by Bell et al. (5). Briefly, mice were infected by gavage with 300 third-stage larvae (L3). Feces were collected from mice. Between 10 and 90 days post-infection, mice were placed on screens above wet paper towels for 24 hours. Feces were collected and mixed with activated charcoal (1:3), set at room temperature for 7-8 days, and L3 collected by the Baerman technique.

Trichinella first-stage larvae (L1) were recovered from muscle tissue of irradiated AO rats by digestion with 1% pepsin in acidified water, as described previously (10). Rats had been infected for at least 28 days prior to euthanasia.

The experimental protocol for immunization was modified from that described by Bell et al. (4). Briefly, rats were infected orally with 250-500 *H. polygyrus* L3. Monoclonal rat IgG2a, IgG2a Fab'2, or NRS IgG (25 mg/rat) was administered by intraperitoneal injection 12 or 13 days later. Antibody was transferred 4 or 24 hours prior to oral infection with 500-1000 *T. spiralis* L1. Rats were euthanized by CO_2 inhalation followed by cervical dislocation 18-24 hours post-challenge, and intestines were collected for worm burden

estimates 18-24 hours post-challenge (1). The mean intestinal larvae burden was reported as a percentage of the dose.

Histology and cytology

Two cm sections of jejunum were collected, fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for three hours, transferred to 70% ethanol, and embedded in paraffin. For mast cell enumeration, sections were stained with Alcian blue (pH. 0.4) and counterstained with Nuclear Fast Red. Eosinophils were counted in tissues fixed in 10% neutral buffered formalin and stained with hematoxylin and eosin (H & E). Mucosal mast cells, and eosinophils were counted in twenty crypt-villus units (CVU) per rat, and the means for rats in treatment groups were reported.

Blood smears were fixed and stained using Protocol Hema 3 stain set (Fisher Scientific Company, L.L.C.; Kalamazoo, MI). A total of 300 white blood cells were counted, and the percent eosinophils calculated. The means for rats in treatment groups were reported. Tissue sections and blood smears were examined on an Olympus BX51 microscope (Melville, NY).

Distribution of larvae in the intestinal tract

Distribution of larvae in the intestine was evaluated as previously described (9). Rats were euthanized 60 to 90 minutes post-challenge. Luminal contents were flushed from the stomach, proximal small intestine, and distal small intestine with 0.85% NaCl, collected into 50 mL polypropylene conical tubes, and held on ice. Larvae trapped in mucus were observed and counted by pressing mucus between glass slides. Larvae free in saline were

considered to have been free in the lumen. Intestines were incubated at 37°C for 5 hrs, and larvae migrating out of the epithelium were counted. Parasites were counted with a dissecting scope (Zeiss, West Germany).

Rat mast cell protease II (RMCP II) measurement

RMCP II was detected in rat sera using the RMCP II ELISA kit purchased from Moredun Scientific Limited (Midlothian, Scotland). Concentrations (ng/ml) in sera were estimated from a standard curve following the manufacturer's instructions and reported as the mean per treatment group.

Complement measurement and depletion

Rats were injected intraperitoneally with 30 IU of cobra venom factor (CVF) (Quidel; San Diego, CA) or an equivalent volume of sterile water, 24 hours prior to oral infection. C3 was measured in serum by radial immunodiffusion assay (RID). Briefly, 2% SeaKem LE agarose (Cambre Bio Science Rockland Inc.; Rockland, ME) was melted in PBS and cooled to 58° C before adding 60 µg of goat anti-rat C3 (MP Biomedicals, LLC.; Solon, Ohio) per mL. Thirty milliliters of agarose mixture were poured into 100 X 15 mm polystyrene square Petri plates (Nalge Nunc International; Rochester, NY), and 3 mm holes were punched evenly across the plate at 1.5 mm spacing. Normal rat serum was serially diluted for a standard curve and 10 µL of serum was delivered to each well. Plates were set in a humidified box for 72 hours before measuring precipitation rings with a 1/60 inch engineering ruler.

Statistical analysis

Parasite burdens and cell numbers were evaluated by analysis of variance (ANOVA) and Scheffe's comparison of means using Statistix (Analytical Software, Tallahassee, FL). Values were considered statistically significant when p was <0.05.

Results

Intestinal mastocytosis induced by H. polygyrus *infection in different rat strains.*

We compared mast cell numbers in the small intestines of AO, PVG, Lewis, BN, and LouM rats that were naïve or had undergone *H. polygyrus* infection, passive immunization, and *T. spiralis* challenge. BN rats had the highest basal mast cell number but responded with a minimal mastocytosis upon infection (Figure 3.1). Lewis rats demonstrated the most dramatic mastocytosis (Figure 3.1). Mastocytosis was limited in PVG and LouM and moderate in AO strain rats. We conducted subsequent studies in Lewis and AO rats.

We titrated the dose of *H. polygyrus* to investigate the effect of that infection on mastocytosis and worm expulsion. Increasing doses of *H. polygryus* L3 induced significant nonspecific expulsion of larvae in Lewis rats (Figure 3.2 A). This correlated with increasing mast cell numbers in the small intestine (Figure 3.2 B). To minimize the confounding effects of nonspecific expulsion induced by oral infection with *H. polygyrus*, we used a dose of 250 *H. polygyrus* L3 dose for subsequent experiments.

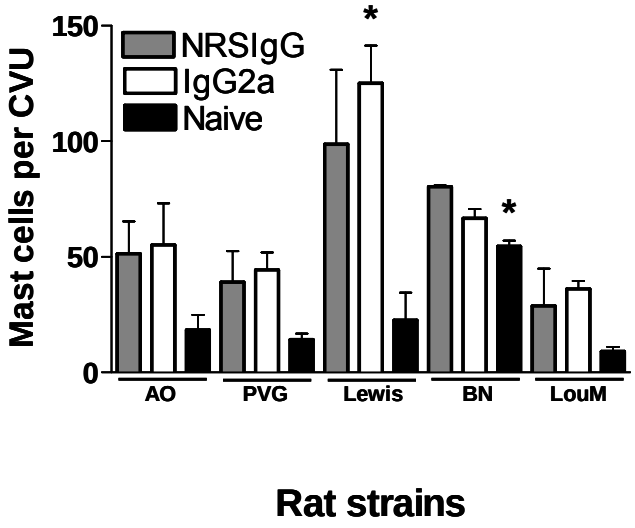


Figure 3.1: Intestinal mastocytosis induced by *H. polygyrus* infection in different strains of rats. Mast cell numbers were quantified in small intestines of naive rats or rats infected with 400 *H. polygyrus*, passively immunized with NRS IgG or anti-tyvelose IgG2a, and infected with *T. spiralis*. Asterisks denote values that were significantly higher ($p < 0.05$) than those of similarly treated rats of other strains. Values reported are the mean +/- 1 SD mast cell per crypt villus unit (n = 3 - 5 rats per group).

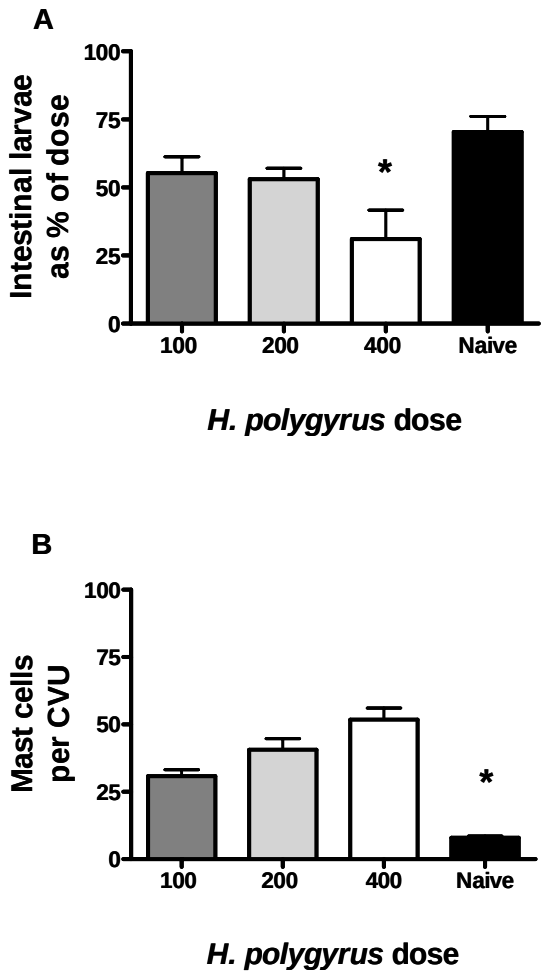


Figure 3.2: Dose response of cross-protection and mastocytosis induced by infection with *H. polygyrus* in Lewis rats. (A) Intestinal worm burdens 24 hours post-infection with *T. spiralis* in Lewis rats given increasing numbers of *H. polygyrus*. (B) Mast cells in intestines of rats described in A. Asterisks denote means that were significantly different from all other groups ($p < 0.05$). Values are reported as mean +/- 1 SD (n = 4 - 5 rats per group).

Characteristics of expulsion by rats passively immunized with IgG2a.

Passive immunization with monoclonal IgG2a of *H. polygyrus* infected Lewis rats prior to challenge resulted in 43% reduction in intestinal worms (Figure 3.3 A). Limited expulsion (21% protection) occurred in the NRS IgG group, possibly due to cross-reactive immunity induced by *H. polygyrus* or to inflamed intestinal environment induced by that parasite. Passively immunized AO rats were not significantly protected when compared to naïve rats (13% protection) (Figure 3.3 B). Mast cell numbers in the small intestine were similar in naïve AO and Lewis rats; however, *H. polygyrus* infection induced a significantly larger mastocytosis in Lewis rats as compared to AO rats (Figure 3.3 C). *H. polygyrus* infection induced a mild blood eosinophilia (Figure 3.3 D) and a significant small intestinal eosinophilia (Figure 3.3 E).

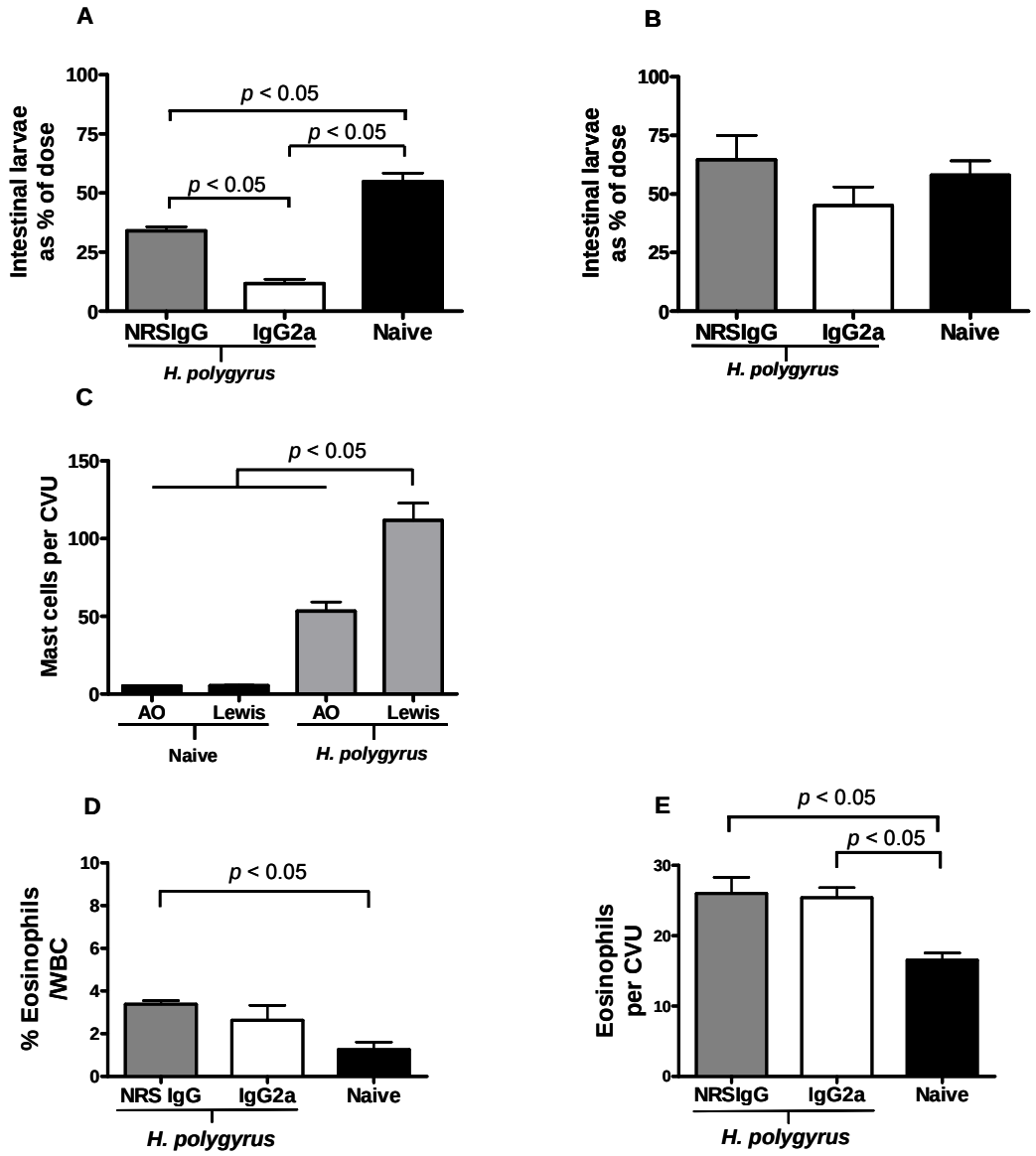
Distribution of larvae in the intestinal tract.

Limited numbers of larvae were entrapped in mucus from H. polygyrus infected rats, regardless of whether they received specific or irrelevant IgG (Figure 3.4 A). Rats passively immunized with IgG2a either excluded larvae from the epithelium or encumbered them within the epithelium (Figure 3.4 B, C), while rats treated with NRS IgG or naïve rats allowed larvae to establish in the epithelium (Figure 3.4 C).

RMCP-II detection in serum.

H. polygyrus infected Lewis rats had significantly elevated RMCP-II serum levels 13 days post-infection (Figure 3.5 A) Lewis rats treated with NRS IgG and IgG2a had elevated RMCP-II serum levels 30 and 90 minutes

Figure 3.3: Passive immunization of *H. polygyrus*-infected Lewis and AO rats with tyvelose-specific monoclonal rat IgG2a. Intestinal worm burdens for (A) Lewis and (B) AO rats (expressed as a percentage of the dose) 24 hours post-challenge with *T. spiralis*. Data in A represent the means of five independent experiments (n = 3 – 8 rats/experiment). Data in B represent the means of three independent experiments (n = 3 – 4 rats/experiment). (C) Mast cells per crypt villus unit in AO and Lewis rats 24 hours post-challenge. (D) Blood eosinophilia in Lewis rats 24 hours post-challenge. (E) Intestinal eosinophilia in Lewis rats 24 hours post-challenge. Bars denote significant differences between groups ($p < 0.05$). Values represent the mean +/- 1 SD (n = 3 - 8 rats per group).


A
Intestinal larvae as % of dose
100
75
50
25
0
p < 0.05
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NRSIgG
IgG2a
Naive
H. polygyrus
B
Intestinal larvae as % of dose
100
75
50
25
0
NRSIgG
IgG2a
Naive
H. polygyrus
C
Mast cells per CVU
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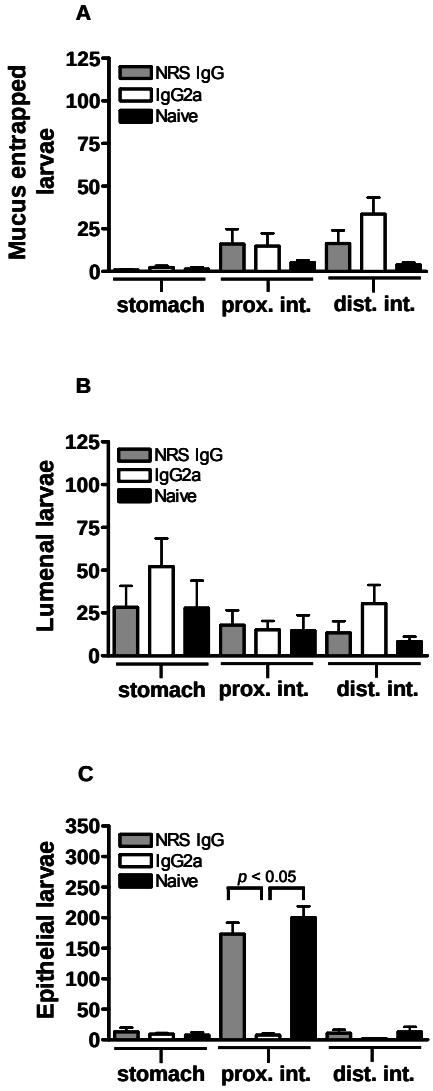

Figure 3.4: Distribution of *T. spiralis* larvae in intestinal tracts of naive and immunized rats. Sixty to 90 minutes post-challenge, rats were euthanized and stomach, proximal intestine, and distal intestine were washed and collected separately. (A) Larvae trapped in mucus. (B) Larvae free in the lumen. (C) Larvae in the epithelium are considered to have successfully infected the host. Values were reported as means +/- 1 SD (n = 4 rats per group). Bars denote statistical significance between groups ($p < 0.05$).

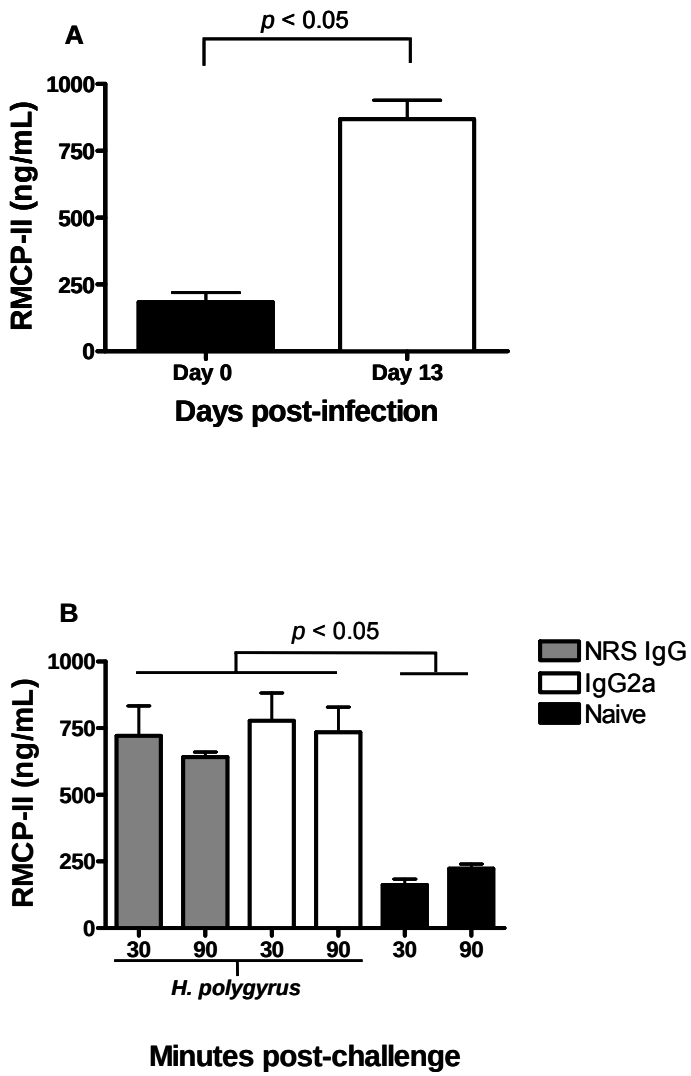

Figure 3.5: Measurement of RMCP-II in sera of immunized or naive Lewis rats following challenge with *T. spiralis*. (A) Lewis rats were bled before (day 0) and after (day 13) *H. polygyrus* infection. (B) Lewis rats infected with *H. polygyrus* and passively immunized with IgG were bled 30 and 90 minutes post-challenge with *T. spiralis*. Values are reported as the mean +/- 1 SD. Bars represent significant differences between groups (n = 4 rats per group).

post-challenge as compared to naïve rats (Figure 3.5 B), but this difference was not greater than *H. polygyrus* infected rats. RMCP-II in sera of IgG2a treated rats was not significantly greater than that of NRS IgG treated rats, demonstrating that IgG2a did not have a detectable effect on mast cell activation.

Fc and C3 dependence of protection mediated by IgG2a.

IgG2a Fab'2 (143 kDa) was found to be free of intact IgG2a (200 kDA) in Western blot. When tested for binding to *T. spiralis* ESA in ELISA, intact IgG2a and Fab'2 fragments bound with similar efficiency; therefore, the fragments were pure and active. Intact IgG2a conferred 44% protection, while NRS IgG and IgG2a Fab'2 conferred 27% and 29% protection, respectively (Figure 3.6 A). Thus the Fab'2 fragments of IgG2a were not protective.

Activation of complement is dependent upon the Fc. RID assay confirmed that C3 was depleted from sera of rats treated with CVF. We detected no precipitin reaction from sera of treated rats as compared with measurable reactions at a 1:16 dilution of sera from control rats. When rats were depleted of C3 and passively immunized with IgG2a, they remained immune to challenge with *T. spiralis* (Figure 3.6 B). Therefore, complement was not essential for protection mediated by IgG2a.

Discussion

Rats respond to a challenge infection with *Trichinella* by expelling 99% of intestinal larvae within a few hours (6, 14). Multiple rat strains display rapid expulsion when challenged after natural infection (3). This immunity can be

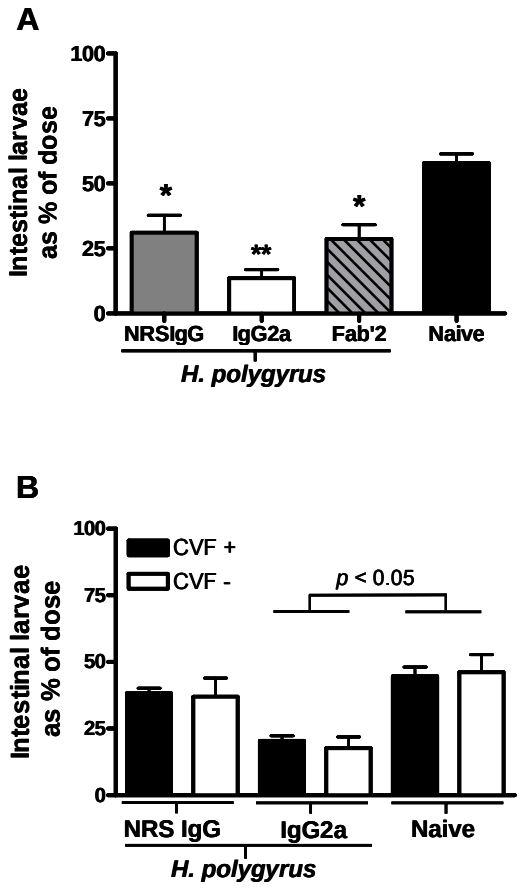

Figure 3.6: The role of the Fc in protective immunity mediated by IgG2a. (A) Rats were infected with *H. polygyrus* and passively immunized with intact IgG2a, Fab'2 fragments of IgG2a or intact control IgG prior to challenge with *T. spiralis*. Unimmunized rats (naive) served as controls. Single asterisk denotes significant differences between NRS and Fab'2 treated groups from naive rats, and double asterisk denotes significant differences between the IgG2a treated group and all other groups ($p < 0.05$). (B) Rats were treated with 30 IU of CVF or equivalent volume of sterile water vehicle. Bars denote significant differences between groups ($p < 0.05$). Values are reported as the mean +/- 1 SD number of worms in the intestine expressed as a percentage of the dose (n = 3 – 8 rats per group).

recreated by passive immunization with IgE or certain IgG isoypes, but only in recipients that have previously received an oral infection with a heterologous nematode, such as *H. polygyrus* or *Nippostrongylus brasiliensis* (4, 13). Since antibody alone is not protective, it has been thought that immune complexes might be interacting with some cellular component of the immune system that is expanded or induced by intestinal inflammation. Prominent cellular changes in the intestine include a goblet cell hyperplasia, mastocytosis and eosinophilia. Mast cells and eosinophils bear Fc receptors, and it is possible that immune complexes might be activating these cells in the inflamed intestine.

We found that *H. polygyrus*-infected Lewis rats that were passively immunized with monoclonal anti-tyvelose IgG2a were protected against challenge with *T. spiralis*. Previous experiments in AO rats passively immunized with IgG1, reproduced rapid expulsion (4); however, we were unable to replicate those results with IgG2a. Our data show that the magnitude of mastocytosis induced by *H. polygyrus* varied among rat strains. This correlated loosely with immunity; however, other inflammatory changes such as eosinophilia were present and may contribute to immunity. Lewis rats mounted the most dramatic mastocytosis in the intestine. When Lewis rats were challenged, we observed some non-specific, or cross-reactive, immunity to *T. spiralis* that was induced by *H. polygryus*. Titration of *H. polygyrus* dose showed that this effect was dose dependent. Since *H. polygyrus* and *T. spiralis* share some antigens (16), it is possible that this protective effect is antigen specific. Alternatively, protection may be due to the innate immune response involving mast cells and elicited by *H. polygyrus*. Primary infection with *T. spiralis* induces intestinal mastocytosis. Mast cell activation occurs

during expulsion of adult worms from the intestine (15), and RMCP-II and other mast cell mediators are detected in the blood (18) of rats only infected with *H. polygyrus*.

Mast cells bear the high affinity IgE receptor (FcεRI) as well as IgG receptors (FcγR). While much is known about the FcεRI on mast cells, less is appreciated about FcγRs or how IgG isotypes, interacting with these FcγRs, might influence mast cell activation. It has been demonstrated *in vitro* that IgG2a binds FcεRI and, upon aggregation by antigen, triggers mast cell degranulation (7). IgG complexes also degranulate mouse mast cells via low affinity FcγRIII receptors; however, this has not been demonstrated in rat mucosal mast cells. Human mast cells are induced by IFN-γ to express the high affinity IgG receptor (FcγRI), which upon aggregation with IgG triggers mast cell degranulation (19). FcγRs on rat mast cells (11, 12) and specifically mucosal mast cells have not been fully characterized nor has their function during parasitic infection been investigated *in vivo*.

Tyvelose-specific IgG1 and IgG2c protect neonatal rats against *T. spiralis* but IgG2a does not (2). Additionally IgG2a does not cause mucus entrapment in passively immunized neonatal rats while the other two isotypes do (8). Passive transfer of IgG1 to *H. polygyrus* infected adult rats does afford protection and promotes mucus entrapment (4). We found that IgG2a does not promote mucus entrapment in *H. polygyrus* infected adult rats. Taken together, these observations support the conclusion that IgG2a functions differently from IgG1 to effect expulsion. We tested the role of the Fc portion of IgG2a in protection and found that Fab'2 fragments do not confer protection. There are several possible explanations for this result. First, IgG2a fragments do not have as long a half life as intact fragments; however, our challenge

experiments were initiated within 4 hours of antibody delivery. Second, the Fc of IgG2a might be activating complement to facilitate rapid expulsion; however, our results show that protection is not dependent on C3. Alternatively IgG2a fragments may not have been distributed in concentrations sufficient to affect immunity. Arguing against this explanation is the increased intestinal permeability, promoted by mast cell activation evident at the time of challenge that should allow fragments to reach the gut lumen. Finally, protection could be dependent upon IgG2a binding to Fc receptors.

Mast cells are activated during *H. polygyrus* infection, indicating that degranulation was not promoted by IgG2a. Passive immunization with IgG2a and challenge with *T. spiralis* did not cause a detectable increase in serum RMCP-II. To determine relevance of mast cells in rapid expulsion, mast cell-deficient rats would need to be tested. We were unable to obtain Ws/Ws strain rats needed to perform these experiments.

We have demonstrated that IgG2a mediates immunity to *T. spiralis* independently of mucus and complement. Our data support a role for the Fc portion of IgG2a or for FcR-bearing cells in protection mediated by IgG2a. The identity and necessity of these cells remains to be determined. Mast cells may facilitate the delivery of antibodies to the epithelium by altering epithelial permeability, but mast cell activation is independent of IgG2a.

Acknowledgements

We thank the animal care staff at the James A. Baker Institute for Animal Health for their expertise, Dr. Gerrie Leslie (Immunology Consultants Laboratory, Inc.) for advice on the C3 RID assay, Dr. Hugh Miller (University of Edinburgh) for advice on RMCP-II, Mr. Linc Adams (Cornell University) for *H. polygyrus* and technical advice, and Ms. Lucy Gagliardo (Cornell University) for technical assistance. This work was supported by the National Institutes of Health grants, AI 14490 and T32 RR0759.

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CHAPTER FOUR

Parenteral infection induces intestinal immunity against *Trichinella spiralis*[*]

[*] Seana M. Thrasher and Judith A. Appleton. Parenteral infection induces intestinal immunity against *Trichinella spiralis*. Manuscript in preparation.

Abstract

Rats previously infected with *Trichinella spiralis* exhibit a dramatic protective immunity to challenge infection that has been called rapid expulsion. A similar immunity is induced when rats are injected intravenously with newborn larvae, an infection protocol that establishes muscle infection in the absence of intestinal infection. In this report, we describe results of experiments designed to further characterize the mechanism of immunity induced by parenteral infection. Infection induced strong IgG1 and IgG2a antibody responses, yet caused no intestinal mastocytosis, goblet cell hyperplasia, or tissue eosinophilia. Nevertheless, mast cells were activated during expulsion as indicated by detection of RMCP-II in serum 90 minutes post-challenge. Furthermore, mucus entrapment of larvae proceeded in a manner similar to that observed in rats immunized by prior oral infection. This model of rapid expulsion demonstrates that parenteral immunization induces a pronounced mucosal immunity in the intestine that engages mast cells.

Introduction

Parasitic nematodes are a persistent global problem for people and livestock. In the context of ongoing concerns regarding drug resistance in nematodes (22), vaccination against parasitic infection has renewed relevance. Understanding immunity to intestine-dwelling nematodes has been advanced by studies in laboratory animals, including *Trichinella spiralis* in rats. Rats infected with *T. spiralis* are protected against secondary infections. This potent mucosal immunity has been referred to as rapid expulsion (11).

Rapid expulsion is a protective immunity against *T. spiralis* that eliminates as many as 99% of intestinal larvae within hours of challenge (11,

17, 29) and is observed exclusively in rats (3). There is substantial evidence that antibody plays a crucial role in protection. Rat pups suckling a *T. spiralis*-infected foster dam demonstrate rapid expulsion, and immunity is conferred by parasite-specific antibodies in the milk (6,5). Passive transfer of parasite-specific monoclonal antibodies is protective as well. While these data provide strong evidence for the role of antibody in suckling rats, passive transfer of monoclonal antibodies or immune serum fail to promote expulsion in weaned rats (35). Passively immunized adult rats demonstrate rapid expulsion only when "primed" by an intestinal infection with an unrelated nematode such as *Heligmosomoides polygyrus* (10) or *Nippostrongylus brasiliensis* (23). It is thought that inflammatory changes in the intestine are required for antibodies to realize their protective function in adult animals.

Mast cells have been implicated in rapid expulsion because intestinal mastocytosis is induced by primary infection with *T. spiralis* (41), and rat mast cell protease-II (RMCP-II) is released coincident with expulsion of adult worms (41) and challenge larvae (34). Compounds released by mast cells during expulsion cause changes in the intestinal environment that may inhibit parasite establishment. RMCP-II (36), prostaglandins (43), serotonin (28), leukotrienes (34), histamine (43), and cytokines (24) affect intestinal secretion (18), permeability (14), and smooth muscle contraction (39). Any of these events may promote worm expulsion, although none of them have been proven to mediate rapid expulsion.

Rats injected intravenously with *T. spiralis* newborn larvae (NBL) develop muscle infections and demonstrate strong immunity to oral infection (5, 12). This immunization protocol circumvents direct stimulation of the many inflammatory changes induced by worms in the intestine. In this report, we

describe results of experiments designed to determine whether immunity induced by parenteral infection is the equivalent of rapid expulsion and to further characterize the mechanism of immunity.

Materials and Methods

Rats and mice

Female Albino Oxford (AO) and Lewis strain rats (8 to 12 weeks old) were bred and maintained in the James A. Baker Institute for Animal Health vivarium. All rodents were housed in accordance with the guidelines of the American Association for Accreditation of Laboratory Animal Care. Blood was collected from tails of rats anesthetized with isoflurane.

Trichinella spiralis

Trichinella spiralis was maintained in irradiated AO rats as decribed by Crum *et al.* (19). Mature first-stage larvae were recovered by digestion with 1% pepsin in acidified water, and newborn larvae (NBL) were recovered from cultures of adult worms as described (5).

Parenteral infections were established by injecting 80,000 NBL into the lateral tail vein. Control groups included naïve, uninfected rats, or rats that had been infected orally with 1,000 L1. Rats were challenged orally with 500 *T. spiralis* L1 60-90 days following the primary infection. Rats were euthanized by CO_2 inhalation followed by cervical dislocation. Intestines were collected for worm enumeration 18 - 24 hours post-challenge unless otherwise specified (4). Muscle burdens were determined by counting larvae freed from carcasses by digestion with pepsin in acidified water. Larval suspensions from

each rat were sampled three times in order to estimate total parasite burden. Parasites were counted with a dissecting microscope (Zeiss, West Germany).

Distribution of larvae in the intestinal tract

In order to evaluate entrapment of intestinal larvae in mucus, rats were euthanized 60 to 90 minutes post-challenge; and the contents of the stomach, proximal small intestine, and distal small intestine were examined as previously described (16). Briefly, intestinal contents were flushed with 0.85% NaCl, collected into 50 mL polypropylene conical tubes, and packed on ice. Larvae trapped in mucus were observed by pressing mucus between glass slides. Larvae free in saline were considered to have been free in the lumen. Intestines were incubated at 37°C for 5 hours, and larvae migrating out of the epithelium were counted.

Histology and cytology

Two cm sections of jejunum were collected, fixed in Carnoy's solution (60% ethanol, 30% chloroform, 10% glacial acetic acid) for three hours, transferred to 70% ethanol, and embedded in paraffin. For mast cell enumeration, sections were stained with Alcian blue (pH 0.4) and counterstained with Nuclear Fast Red. Alternatively, goblet cells were enumerated in sections stained with hematoxylin and eosin (H & E). Eosinophils were counted in tissues fixed in 10% neutral buffered formalin and stained with H & E. Mucosal mast cells, goblet cells, and eosinophils were counted in twenty crypt-villus units (CVU) per rat, and the mean number per CVU for rats in treatment groups were reported.

Blood smears were fixed and stained using Protocol Hema 3 stain set (Fisher Scientific Company, L.L.C.; Kalamazoo, MI). A total of 300 white blood cells were counted, the percent eosinophils calculated, and reported as a mean for each treatment group. Tissue sections and blood smears were examined on an Olympus BX51 microscope (Melville, NY).

Measurement of RMCP-II in serum

RMCP-II was detected in rat sera using the RMCP-II ELISA kit purchased from Moredun Scientific Limited (Midlothian, Scotland)**.** Concentrations (ng/ml) in sera were estimated from a standard curve and reported as the mean per treatment group.

ELISA for rat IgG isotypes and IgE

Parasite-specific immunoglobulins were quantified by ELISA. Antibodies were detected with biotinylated monoclonal mouse anti-rat IgG1 (RB11/39), IgG2a (RG7/1), IgG2b (RG7/11) (37), or IgE (MARE-1). Antibodies were biotinylated with N-hydroxysuccinimidobiotin (Sigma; St Louis, MO) in bicarbonate buffer (pH 9.5; 120 µg biotin per 1 mg of antibody) for 4 hours at room temperature. Biotin-conjugated anti-rat IgG2c and anti-rat IgM antibodies were purchased (BD Pharmingen; San Diego, CA). Standard curves for IgG1, IgG2a, IgG2b, IgG2c and IgM utilized parasite-specific monoclonal antibodies 9D4, 18H1, 10G11, 9E6 (7), and 305D9 (15), respectively.

All incubations were carried out for 1 hour at room temperature. 96-well polyvinyl plates were coated with 1µg/mL of *T. spiralis* excretory-secretory antigen (ESA) (8) diluted in 10% Dulbecco's PBS (DPBS) and incubated

overnight at 4°C. Plates were blocked with DPBS containing 0.2% gelatin. Sera from rats were diluted 1:6,000 for measuring IgG1 and IgG2a, 1:100 or 1:500 for IgG2b, 1:2500 for IgG2c, and 1:500 or 1:2500 for IgM. Biotin-conjugated anti-rat isotypes were diluted in DPBS containing 0.1% gelatin and 10% normal mouse serum and used at the following concentrations: IgG1, IgG2a, IgG2b (1 µg/mL), IgG2c and IgM (0.25 µg/mL). Streptavidin-horseradish peroxidase (0.2 µg/mL), (BD Pharmingen; San Diego, CA) was diluted in DPBS containing 0.1% gelatin. The plates were developed for 5 minutes with tetramethylbenzidine (TMB) (KPL Laboratories, Gaithersburg, MD) and stopped with 1 M H_3PO_4. Absorbance (450 nm) was measured with an ELISA plate reader (Bio-Tek Instruments, Winooski, VT). In order to measure total serum IgE, ELISA plates were coated with anti-rat IgE mAb A2 (5 µg/ml), rat myeloma IgE 162 served as a standard, rat sera were diluted 1:100, and biotin-conjugated MARE-1 (5 µg/mL) was used as the detection reagent.

Complement measurement and depletion

C3 was measured in serum by radial immunodiffusion assay (RID). Briefly, 2% SeaKem LE agarose (Cambre Bio Science Rockland Inc.; Rockland, ME) was melted in PBS and cooled to 58°C before adding 60 µg of goat anti-rat complement C3 (MP Biomedicals, LLC.; Solon, Ohio) per mL of agarose mixture. Thirty milliliters of agarose mixture were poured into 100 X 15 mm polystyrene square Petri plates (Nalge Nunc International; Rochester, NY), and 3 mm holes were punched evenly across the plate at 1.5 mm spacing. Normal rat serum was serially diluted for a standard curve. Plates

were set in a humidified box for 72 hours before measuring precipitation rings with 1/60 inch engineering ruler.

Rats were injected intraperitoneally with 30 IU of cobra venom factor (Quidel; San Diego, CA) or an equivalent volume of sterile water 24 hours prior to oral challenge with *T. spiralis*. C3 was assayed in serum collected from rats 18 - 24 hours post-challenge.

Statistical analysis

Parasite burdens and cell numbers were evaluated by analysis of variance (ANOVA) and Scheffe's comparison of the means or by Student's t test using Statistix (Analytical Software; Tallahassee, FL). Values were considered statistically significant when p was <0.05.

Results

Life-stage specificity of immunity induced by parenteral infection.

To determine whether protection conferred by parenteral infection was specific for first-stage larvae, we evaluated intestinal burdens 24 and 72 hours following oral challenge (Figure 4.1). Parenterally infected AO rats expelled 86% of larvae by 24 hours. No additional larvae were expelled between 24 and 72 hours, demonstrating that immunity was specific for L1 and consistent with rapid expulsion. Recovery of larger numbers of worms from naïve rats at 72 versus 24 hours was likely the result of the failure of molting larvae, present at 24 hours post-infection, to emerge from the epithelium (35).

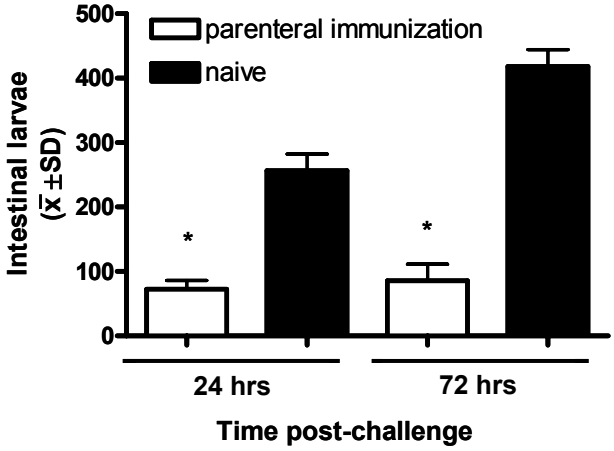


Figure 4.1: Specificity of immunity induced by parenteral infection. AO rats injected with 80,000 *T. spiralis* NBL were challenged orally two months later with 500 *T. spiralis* first-stage larvae. Intestinal worm burdens were determined 24 and 72 hours post-challenge and are reported as the mean +/- 1 SD (n = 4 - 5 rats per group). Asterisks denote significant differences ($p < 0.05$) between immunized and naive rats.

Entrapment of larvae in mucus during expulsion.

Mucus entrapment of larvae is a hallmark of rapid expulsion. We compared two strains of rats, AO and Lewis, because the protection conferred by oral infection with a heterologous nematode was significantly different in the two strains (see Chapter 3). Both parenterally and orally infected rats demonstrated mucus entrapment of intestinal larvae at 60 minutes post-oral challenge (Figure 4.2 A, D) and excluded larvae from the epithelium (Figure 4.2 B-F). Naive rats did not entrap significant numbers of larvae in mucus (Figure 4.2 A, D) and allowed large numbers of larvae to enter the epithelium (Figure 4.2 C, F). Larvae free in the lumen were similar for all groups (Figure 4.2 B, E). No differences were observed between strains, nor were the kinetics of entrapment and exclusion different between orally and parenterally immunized rats.

Effects of parenteral infection on intestinal mastocytosis, goblet cell hyperplasia, and eosinophilia.

Intestinal mastocytosis and goblet cell hyperplasia are prominent in orally infected rats (10, 32). Lewis and AO strain rats differ markedly in the magnitude of mastocytosis induced by nematode infection (see Chapter 3). Both AO and Lewis rats demonstrated rapid expulsion following parenteral immunization and challenge (Figure 4.2 & 4.3 A, F). When parenterally infected, neither strain exhibited appreciable intestinal mastocytosis (Figure 4.3 B, G) or goblet cell hyperplasia (Figure 4.3 C, H). Blood eosinophilia was evident 2 weeks post-oral infection in Lewis rats (Figure 4.3 I), and at 2, 4, and 6 weeks post-oral infection of AO rats (Figure 4.3 D). In both strains,

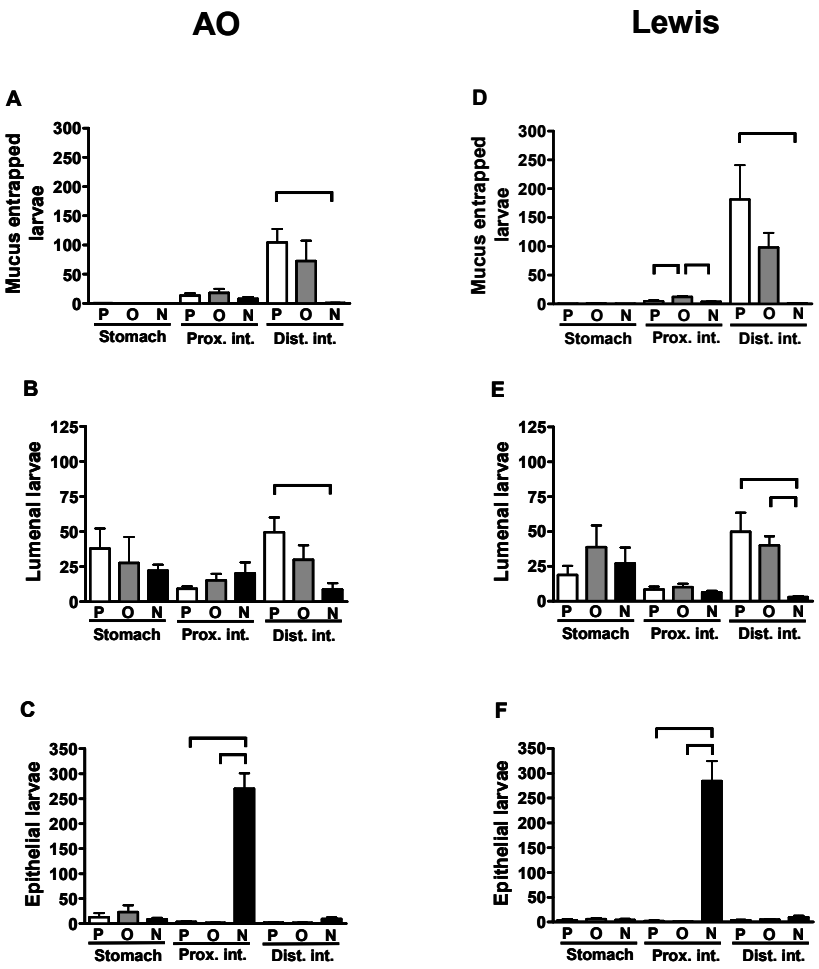
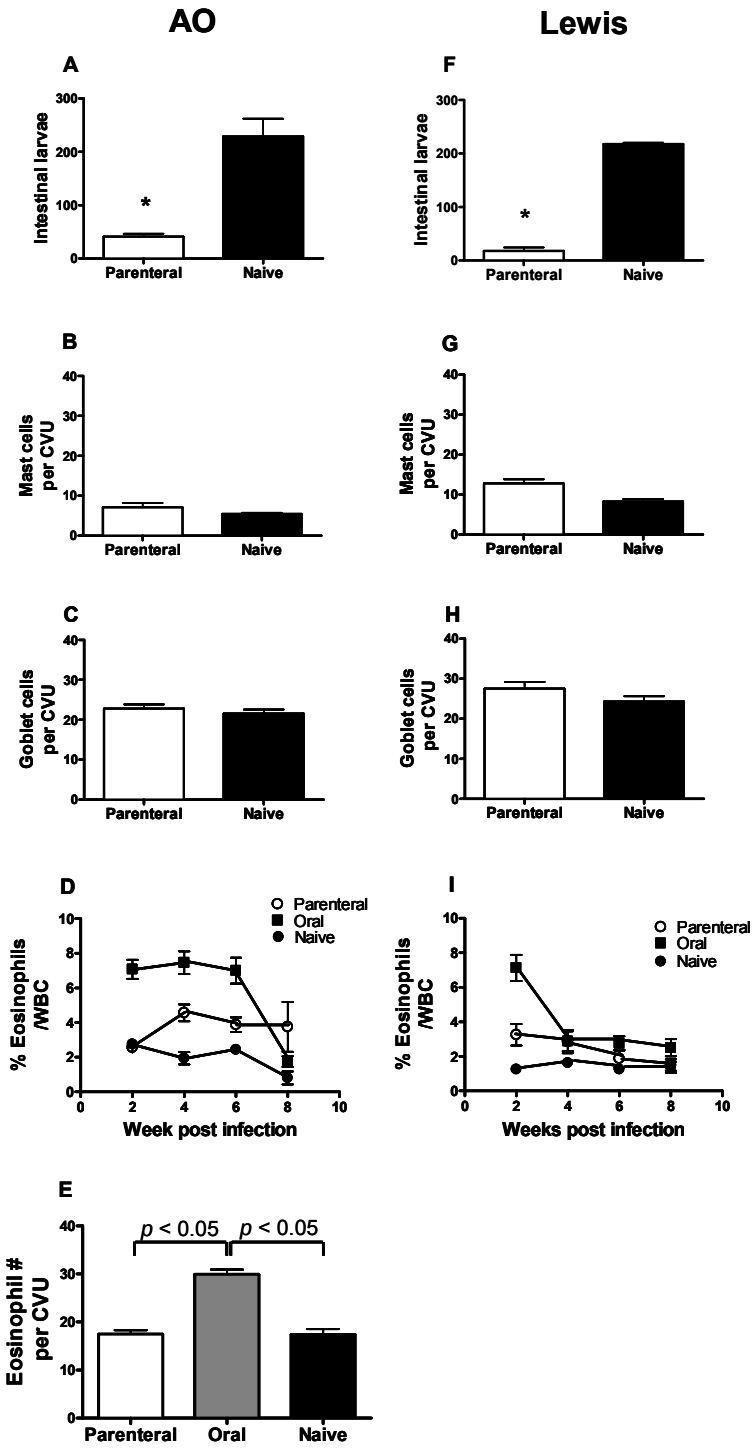


Figure 4.2: Distribution of larvae in parenterally immunized AO (A - C) and Lewis (D - F) rats. P = parenteral infection, O = oral infection, and N = naive. Sixty to 90 minutes post-challenge, rats were euthanized and stomach, proximal small intestine, and distal small intestine contents were washed and collected separately. Worms free in saline were counted as lumenal worms (B, E). Worms that migrated into saline during 5 hours incubation at 37°C were counted as epithelial worms (C, F). Bars denote significant differences ($p < 0.05$) between groups. Values reported are the means +/- 1 SD (n = 4 - 5 rats).

Figure 4.3: Intestinal mastocytosis, goblet cell hyperplasia, eosinophilia or peripherial blood eosinophilia in parenterally infected or naïve AO (A - E) or Lewis rats (F - I). Values shown are means +/- 1 SD for treatment group (n = 4 – 16 rats per group). Asterisk denotes significant differences ($p < 0.05$) between groups.


AO
Lewis
A
F
Intestinal larvae
300
200
100
0
*
Parenteral
Naive
B
G
Mast cells per CVU
40
30
20
10
0
C
H
Goblet cells per CVU
D
I
% Eosinophils /WBC
Parenteral
Oral
Naive
Week post infection
Weeks post infection
E
Eosinophil # per CVU
p < 0.05
p < 0.05
Oral

eosinophilia had resolved by the time of challenge (Figure 4.3 D, I). A modest blood eosinophilia was demonstrated at 4 and 8 weeks post-parenteral infection in AO rats. Eleven weeks post-infection, eosinophils were numerous in the intestines of orally infected rats but not parenterally immunized rats (Figure 4.3 E).

Dectection of RMCP-II in serum following challenge of parenterally and orally infected rats.

Mast cell degranulation and RMCP-II release occurs during rapid expulsion induced by prior oral infection (34). Despite the absence of detectable mastocytosis in the small intestine, parenterally immunized AO rats had significantly elevated serum RMCP-II at 90 minutes post-challenge (Figure 4.4 A). Challenge of naïve rats did not induce release of RMCP-II into the blood. Parenteral infection did not cause an elevation of RMCP-II in blood; rather, serum RMCP-II in infected Lewis rats at 2, 4, and 6 weeks post-infection was equivalent to values in naïve rats (Figure 4.4 C). AO rats had significantly reduced RMCP-II concentrations in sera at 6 weeks post-infection when compared to naïve rats (Figure 4.4 B). Orally infected Lewis rats initially had elevated RMCP-II concentrations 2 weeks post-infection, but this resolved by 4 weeks and increased again 1 hour post-challenge (Figure 4.4 C).

Isotype profiles of antibody responses in parenterally and orally infected rats.

Antibody production in rats orally infected with *T. spiralis* has been documented, but not quantified (4). Because antibody contributes significantly to rapid expulsion of *T. spiralis* in suckling rats and has been implicated in immunity in adult rats, we quantified the isotype response to parenteral

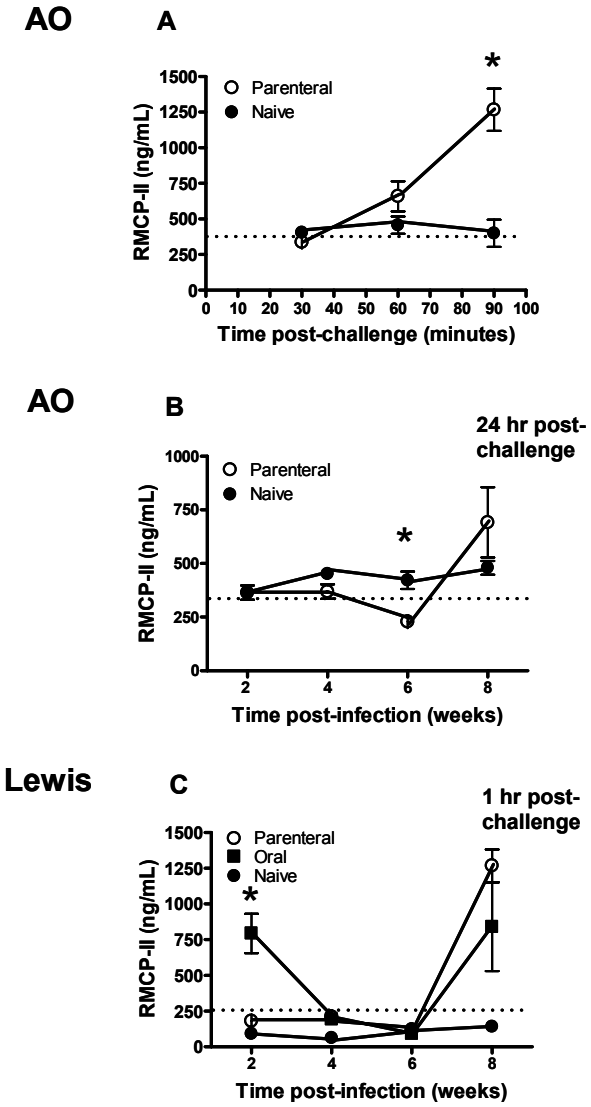


Figure 4.4: Detection of serum RMCP-II during oral or parenteral infection and rapid expulsion. RMCP-II was measured by ELISA. (A) Detection of serum RMCP-II following oral challenge of parenterally infected or naïve AO rats. (B) Parenterally infected AO rats and (C) parenterally and orally infected Lewis rats were bled at 2, 4, 6, and 8 weeks post infection. Dotted line represents RMCP-II concentrations in sera collected from unchallenged naive rats. Values are reported as the mean +/- 1 SD (n = 4 – 8 rats per group). Asterisks denotes significant differences ($p < 0.05$).

infection (Figure 4.5). The most dramatic differences between orally and parenterally infected rats were in parasite-specific IgG2a and total IgE. Parenterally infected rats produced significantly more IgG2a and IgG1 and significantly less IgE compared to orally infected rats (Figure 4.5 C).

Effect of complement depletion on rapid expulsion in parenterally infected rats.

Since parenteral infection induced antibodies that are capable of fixing complement (IgG1 and IgG2a), we tested the role of complement in rapid expulsion. RID assay confirmed that C3 was depleted from sera of rats treated with CVF. We detected no precipitin reaction from sera of treated rats as compared with measurable reactions at a 1:16 dilution of sera from control rats. Treatment with CVF had no effect on rapid expulsion and did not alter parasite establishment in naïve rats (Figure 4.6).

Discussion

Rats respond to oral challenge infection with *T. spiralis* by expelling 99% of first-stage larvae within a few hours (11). Prominent cellular changes induced in the intestine by a primary infection include mastocytosis, goblet cell hyperplasia, and eosinophilia (10). Mast cell activation releases inflammatory mediators, such as histamine, serotonin, prostaglandins, and proteases, that are measurable in serum during rapid expulsion (42), and cause increased intestinal secretion, permeability, and muscle contraction. However, pharmacologic inhibition of various mast cell mediators and the consequent decreases in intestinal fluid secretion, fail to impede worm expulsion (13, 42). This demonstrates that secretion is not the mechanism of expulsion. Nevertheless, other mast cell mediators and their effects may promote rapid

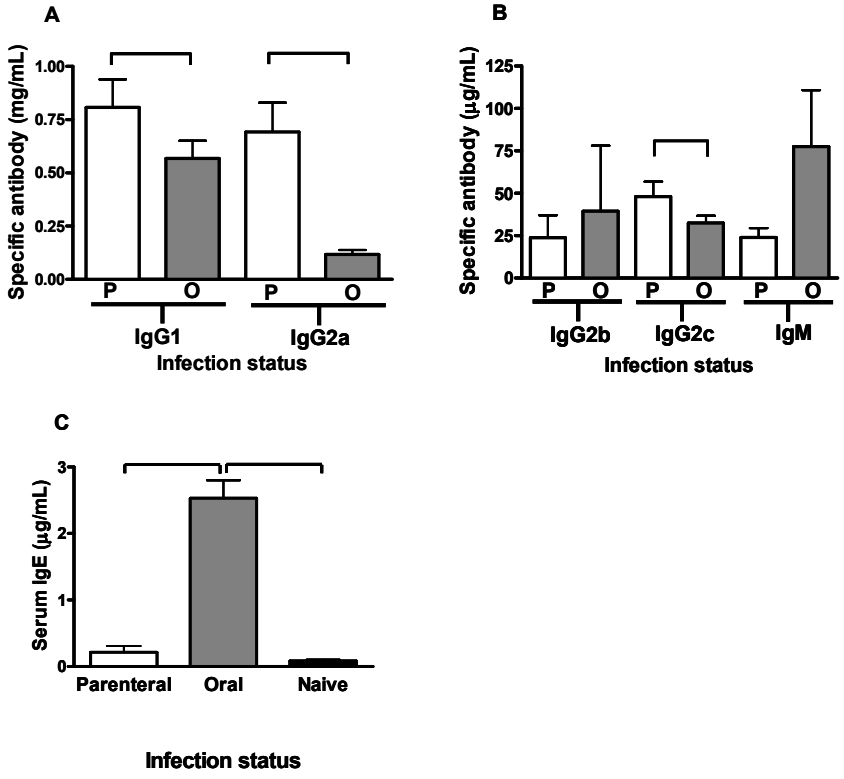


Figure 4.5: Antibody isotypes produced in response to infection with *T. spiralis*. Antibodies of each isotype were quantified in sera of AO rats that were orally infected with 1,000 *T. spiralis* ML (grey columns, O) or parenterally immunized with 80, 000 *T. spiralis* NBL (white columns, P). Sera were collected 8 weeks following oral infection and 10 weeks following parenteral immunization. IgG1, IgG2a, IgG2b, IgG2c, and IgM concentrations reflect antigen-specific antibodies (A, B), while IgE concentrations are the total serum IgE (C). Values are reported as the mean +/- 1 SD (n = 5 rats per group). Bars represent significant differences between groups ($p < 0.05$).

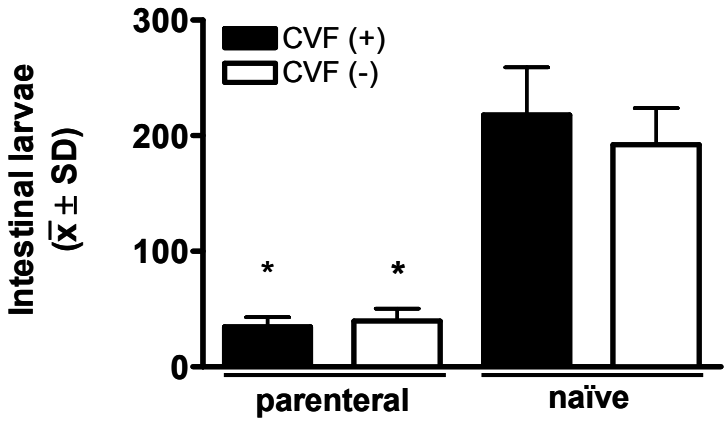


Figure 4.6: Effect of C3 depletion on rapid expulsion induced by parenteral infection. AO rats were treated with 30 IU of CVF or with equivalent volume of sterile water. Values are reported as the mean +/- 1 SD (n = 4 rats per group). Asterisks mark significant differences between groups ($p < 0.05$).

expulsion. In order to eliminate inflammatory and architectural changes induced by a primary oral infection,that may not contribute to rapid expulsion, we set out to characterize immunity that is induced by parenteral infection.

Parenteral infection with *T. spiralis* NBL induced immunity that shared three prominent features of rapid expulsion. Immunity was specific for the infectious first-stage larva (5), mucus entrapment occurred within 60 minutes of challenge (16), and mast cells were activated during expulsion (34). Immunity conferred by parenteral infection is consistently robust and mirrors rapid expulsion observed after natural infection. Immunity induced by models employing oral immunizations do not reproduce the magnitude of protection demonstrated here (10, 23, 1).

Rapid expulsion is specific for the infectious first-stage larva. In suckling rats, immunity is provided by passive transfer of antibodies specific for tyvelose (7). Tyvelose is a dideoxyhexose occupying a terminal position on multi-antennary N-glycans (21). The glycans modify glycoproteins that are synthesized exclusively by L1. Within 6-8 hours of entry into the intestine, first-stage larvae molt, and specific immunity conferred by antibodies to tyvelose no longer functions. Our data demonstrated that rapid expulsion induced by parenteral immunization occurs within hours and is specific for L1; however, oral and parenteral infection induced distinctive antibody isotype profiles. Oral infection induced strong IgG1 and IgE responses with little IgG2a. In contrast, parenteral immunization induced elevated IgG1 and IgG2a and little IgE (40). This modified Th2 response is often observed in chronic helminth infections. IgG2a and IgE share functional properties and may perform the same role in each model of immunity.

A second feature of rapid expulsion is the immediate entrapment of larvae in mucus (10). Previous studies have shown that IgG1, IgG2a, and IgG2c promote mucus entrapment of larvae *in vitro* (15), while only IgG1 and IgG2c cause mucus entrapment *in vivo* (16). Mucus entrapment alone or in coordination with antibody (16, 15, 31), along with goblet cell hyperplasia (32, 33) is known to play a role in rapid expulsion of *N. brasiliensis* in the rat. Mucus entrapment occurs during rapid expulsion of *T. spiralis* but may not be necessary for expulsion (9). Entrapment is reversible in weaned rats (35), and we have concluded previously that it is not essential to rapid expulsion. Both routes of infection induced a strong IgG1 response. In passively immunized suckling rats, IgG1 induces dramatic entrapment of larvae in mucus (15, 16). We speculate that mucus entrapment in parenterally infected rats may be mediated by tyvelose-specific IgG1.

Finally, mast cells were activated during rapid expulsion in parenterally immunized rats, as demonstrated by detection of RMCP-II in serum 60-90 minutes post-challenge. RMCP-II is unique to mucosal mast cells. Unlike orally infected rats, mast cell activation induced in parenterally infected rats occurred without intestinal mastocytosis. We speculate that T cell activated by chronic muscle infection may alter the intestinal environment. Larvae colonized the tongue in parenterally infected rats, and the tongue is part of the mucosal associated lymphoid tissue (MALT). T cells, activated in the cervical lymph nodes (CLN) by larvae that colonize the tongue, may migrate to the intestine and influence cells resident in the intestine. In humans mast cell-T cell cross-talk mediated by OX40-OX40L has been demonstrated within the tonsils (26) and OX40L has the ability to induce naïve $CD4^+$ T cell

differentiation to Th2 cells. It is possible that activated T-cells migrating to the intestine influence mast cell activation despite the absence of mastocytosis.

Rats demonstrate a pronounced eosinophilia in response to oral infection with *T. spiralis* (20). We found that parenteral immunization induced a moderate blood eosinophilia in AO rats, but not in Lewis rats. Intestinal eosinophilia was not evident in either strain. Therefore, expanded numbers of intestinal eosinophils is not essential for rapid expulsion.

Experimental designs used for modeling rapid expulsion have utilized oral infection with heterologous nematodes, such as *H. polygyrus* or *N. brasiliensis,* combined with passive transfer of parasite-specific serum, or monoclonal antibodies (13, 10, 23). Protection conferred in these systems is never as profound as that seen in natural infection or in parenteral immunization. Immunity conferred by *H. polygyrus* infection with passive transfer of antibody provides protection levels of only 65-74% (10). Protection induced by parenteral immunization was 92-95%, nearly equivalent to 99% protection provided by natural infection. In addition, this protection was demonstrable between 8 and 12 weeks and longer post-immunization, a period similar to that associated with oral infection.

In another model, $OX38^+$, $OX8^-$, $OX22^-$ T helper cells isolated from the thoracic duct of *T. spiralis* infected rats were transferred with either immune serum or antibody to naïve rats (1). This model achieved only 40-60% protection. Interestingly, transfer of $OX22^-$ cells induced intestinal eosinophilia while transfer of $OX22^+$ cells induced mastocytosis. Since $OX22^+$ did not protect rats from a challenge infection, it was concluded that mast cells did not play a role in expulsion. Mast cell activation was not assayed in those experiments and, as we have demonstrated here, mast cell activation can

occur in the absence of mastocytosis. Parenteral immunization is an advantageous model for studying rapid expulsion because it not only eliminates the confounding effects of intestinal inflammation but also confers consistently robust protection.

Other studies of immunity to a primary infection with *T. spiralis* have implicated alterations in gastrointestinal motility in expulsion. In these experiments, $CD4^+$ T cells promoted muscle hypertrophy and increased contractility leading to parasite expulsion (38). Cytokines, particularly TGF-β1, upregulate COX-2 and PGE_2 which maintain muscle hypercontractility that persists as long as 6 weeks post-infection and in the absence of ongoing mucosal inflammation (2). The lack of intestinal inflammation and longevity of muscle hypercontractility make this an attractive candidate mechanism for expulsion induced by parenteral immunization. It would be informative to investigate whether persistent muscle hypercontractility occurs as a result of parenteral infection, and whether it persists in the rat long enough to account for immunity observed 8 to 12 weeks post-immunization.

Our data show that parenteral infection stimulates a potent immune response in the intestine. It is possible that NBL, migrating through the lung, induced a mucosal immune response that was transferred to the intestine. It is also possible that muscle larvae residing in the tongue provide an antigenic stimulus that directs lymphocytes to other mucosal sites. Mucosal lymphocytes up-regulate homing molecules, such as α4β7 or αEβ7 and CCR9, that facilitates lymphocyte migration to the intestine (27). Alternatively, since parenteral infection establishes a persistent muscle infection (60 days in our experiments), systemic activation of lymphocytes might also somehow induce trafficking to mucosal sites. Recent studies suggest that parenteral

vaccination can lead to mucosal immunity by up-regulating adhesion molecules specific for mucosal tissues (25, 30). Our model provides an excellent system for further investigation of systemic priming of mucosal immunity.

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CHAPTER FIVE

Summary and conclusions

The intention of this investigation was to clarify the role of mast cells in rapid expulsion of *Trichinella spiralis* from the intestine of the rat. Rapid expulsion is a protective immunity against infection with *T. spiralis* that eliminates as many as 99% of intestinal larvae within hours of challenge and is observed exclusively in rats (1, 12). There is an extensive body of research characterizing this immune response, but a comprehensive mechanism of immunity has not been defined. It is known that rapid expulsion is specific for the infectious first stage larva (L1), and the kinetics of the immune response is similar to a Type I hypersensitivity reaction in that it is initiated within minutes and completed within hours (2, 9). Mucus entrapment of larvae is a hallmark of rapid expulsion; however, mucus entrapment is reversible (7, 8, 16) and not essential to the process (3). Antibodies against the highly immunogenic sugar, tyvelose, that modifies the L1 ESA have been shown to participate in rapid expulsion (4). The requirement for anti-tyvelose antibodies proves the L1 specificity of the immune response. Additionally, it has been observed that an inflammatory infiltrate in the intestine is necessary for passive transfer to be effective in adult rats but not neonatal rats (4). Primary infection with *T. spiralis* induces profound intestinal mastocytosis, eosinophilia, and goblet cell hyperplasia. Parasite specific antibodies have the potential to interact directly with any of these cell types via Fc receptors, yet this has not been directly documented. Because mastocytosis is such a prominent feature of *T. spiralis* infection, many studies of rapid expulsion have focused on the effects of mast cell mediators on expulsion. While it is documented that mast cells are activated during rapid expulsion (13), pharmacologic inhibition of several mast cell mediators have failed to impede worm expulsion (5). 5-hydroxy-tryptamine (5-HT) is one exception. Inhibition of 5-HT did impair expulsion

(23), and it was speculated that this was due to the effect of 5-HT on intestinal secretion. However, changes in intestinal Cl^- secretion are not essential (23). Smooth muscle hypertrophy and hypercontractility occur as a result of parasite induced intestinal inflammation and certainly could contribute to worm expulsion (21); however, immunity conferred by *T. spiralis* infection is long-lived, and it is likely that these smooth muscle changes do not persist for the same period. The documented importance of antibodies and the apparent need for intestinal inflammation to effect rapid expulsion, support a hypothesis that tyvelose-antibody complexes activate mast cells via Fc receptors.

This hypothesis was tested in a model *in vitro* system that assays binding and activation of mucosal mast cells by immune complexes. Bone marrow derived mast cells (BMMC) from the rat are functionally and biochemically very similar to mucosal mast cells (11). Experiments described in Chapter Two confirmed that BMMC displayed a homogenous mucosal phenotype that was superior to other mast cell models: peritoneal cells, RBL-2H3 cells, and mouse BMMC. BMMC contained large, distinct granules that bound Alcian blue, a dye that is specific for mucosal mast cells in the rat. Additionally, BMMC contained rat mast cell protease-II (RMCP-II), a protease found exclusively in mucosal mast cells. In comparison to RBL-2H3 cells, BMMC contained twice the amount of β-hexosaminidase, an enzyme released by mast cells when they degranulate. The mast cell specific ganglioside GD_{1b} was present on the surfaces of both RBL-2H3 and BMMC. Thus, rat bone marrow cells cultured with SCF and IL-3 displayed a mucosal mast cell phenotype.

Comparison of immune complex binding by BMMC and RBL-2H3 revealed that isotype specificity of FcR was the same on both cells types.

Immune complexes containing IgE and IgG2b bound exclusively to the FcεRI and FcγRII, respectively, while those containing IgG1 and IgG2a bound both receptors. IgG2c immune complexes did not bind to the surfaces of either cell type. These flow cytometric studies revealed that surface expression of the inhibitory receptor, FcγRII, on BMMC was four times that of RBL-2H3 cells, while the surface expression of the activating receptor, FcεRI, on BMMC decreased four-fold compared to RBL-2H3 cells. Thus the potential activation/inhibitory ratio of FcRs on BMMC is much lower than for RBL-2H3 cells (14). Stimulation with IgE or IgG2a containing immune complexes induced RBL-2H3 degranulation regardless of whether they were complexed with parasite antigen or DNP-BSA (6); however, IgG2a immune complexes of either specificity did not significantly stimulate BMMC. Structural differences between parasite antigens and DNP-BSA did not account for the lack of BMMC activation, as IgG2a-DNP-BSA also failed to elicit a strong response. It has been reported that tyvelose bearing glycoproteins bind to the surfaces of a rat mast cell line, HRMC. Such binding may affect the responsiveness of mast cells to FcR engagement. We tested surface binding of the two parasite antigens, ESA and *T. spiralis* crude antigen (cAg), used to form immune complexes. ESA did not bind to cell surfaces, but cAg bound to both BMMC and RBL-2H3 cells. Incubating ESA and cAg with the cells prior to stimulation with IgE-DNP-BSA did not affect degranulation. Thus, BMMC are likely refractory to IgG stimulation because of their high surface expression of FcγRII that contributes to a low A/I ratio on these cells.

FcR expression on cells is known to be influenced by cytokines. For example, IFN-γ causes human mast cells to express FcγRI (15). Treatment of B-cells with IL-4 has been shown to decrease FcγRII expression (18). In

addition, treatment of human monocytes with either Th1 or Th2 cytokines dramatically alters both activating and inhibitory Fc gamma receptors on the cell surface (17). It would be informative to grow BMMC in the presence of different cytokines known to be upregulated in parasitic infection, such as IL-4,IL-13, IL-9, IL-10, or TGF-β, and then assay FcR expression and activation by IgG immune complexes. Alternatively, one could use RNAi to eliminate the FcγRII receptor prior to testing activation by IgG immune complexes. Attempts to block this receptor with a specific antibody did not effect IgG2a stimulation of BMMC or RBL cells (data not shown).

IgG2a is protective when used for passive immunization of adult rats in experimental models of rapid expulsion. We decided to study its mechanism of action *in vivo*, using *Heligmosomoides polygyrus* infection together with passive immunization to induce rapid expulsion (4). By testing several rat strains in this model, we found that the potency of expulsion correlated with intestinal mastocytosis. We chose Lewis rats for our investigations because this strain displayed pronounced rapid expulsion and mounted the greatest intestinal mastocytosis. Titration of the *H. polygyrus* dose correlated with nonspecific immunity to *T. spiralis,* either due to cross-reactive antibodies or to the hostile environment generated in the intestine. To eliminate the confounding effects of this nonspecific immunity, we used a lower dose of *H. polygyrus* for our investigations. We concluded that IgG2a did not cause entrapment of larvae in mucus. IgG2a mediated protection occurred in the absence of mast cell activation above that induced by *H. polygyrus* infection alone; however, IgG2a-mediated protection was dependent on the Fc portion of the molecule. Protection did not require complement and the results suggest that IgG2a may interact with FcR-bearing cells. Additional studies

using Ws/Ws rats that are deficient in mast cells or rat treatment with the mast cell stabilizer, doxantrazole, would be useful to determine whether or not IgG2a might be directly interacting with mast cells. We were unable to obtain these rats. The development of knock-out and transgenic rats would also be advantageous to these investigations; however, it will be some time before such laboratory animals become available.

The *H. polygyrus* model of rapid expulsion has many disadvantages. The extensive intestinal changes due to inflammation makes it difficult to determine what part of the immune response is specifically promoting rapid expulsion. Furthermore, the protection provided by passive immunization with antibodies in this model is limited and the model does not replicate all known parameters of rapid expulsion induced by natural infection. Because of these disadvantages, we decided to characterize another model of rapid expulsion, that induced by parenteral infection.

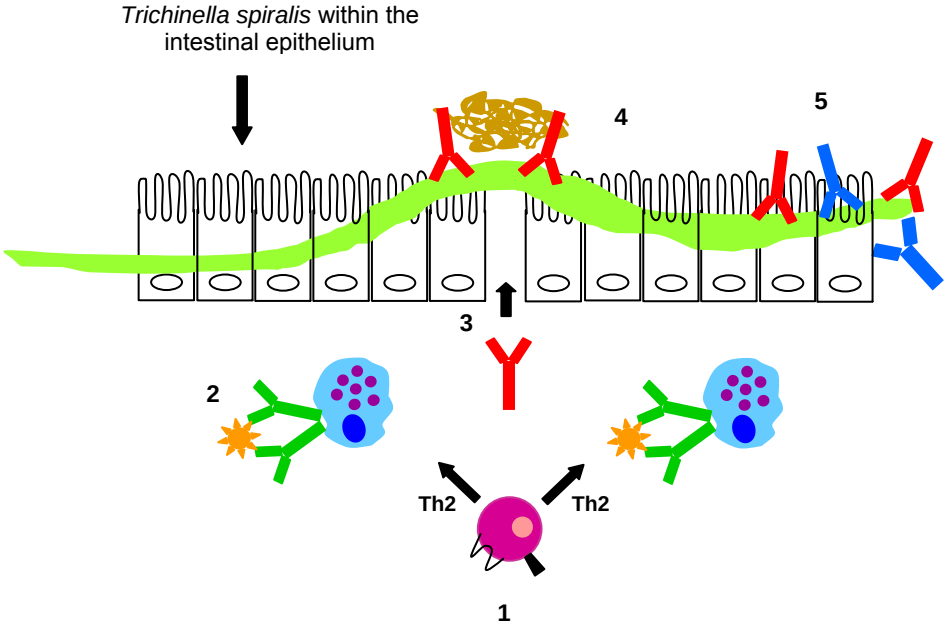
Parenteral infection with *T. spiralis* newborn larvae (NBL) elicits a protective response that is equivalent to that of natural infection. We confirmed that protection provided by parenteral immunization was specific for the infectious first-stage larva and displayed kinetics consistent with rapid expulsion. Like rapid expulsion induced by oral infection with *T. spiralis*, parenteral infection promoted mucus entrapment of larvae. Parenteral infection did not cause eosinophilia, mastocytosis, or goblet cell hyperplasia of the small intestine. Thus parenteral infection eliminated the confounding inflammatory changes in the intestine due to oral infection. Despite the absence of intestinal mastocytosis, mast cells were activated coincident with expulsion. It would be informative to treat parenterally infected rats with doxantrazole, a mast cell stabilizer, prior to challenge to determine whether

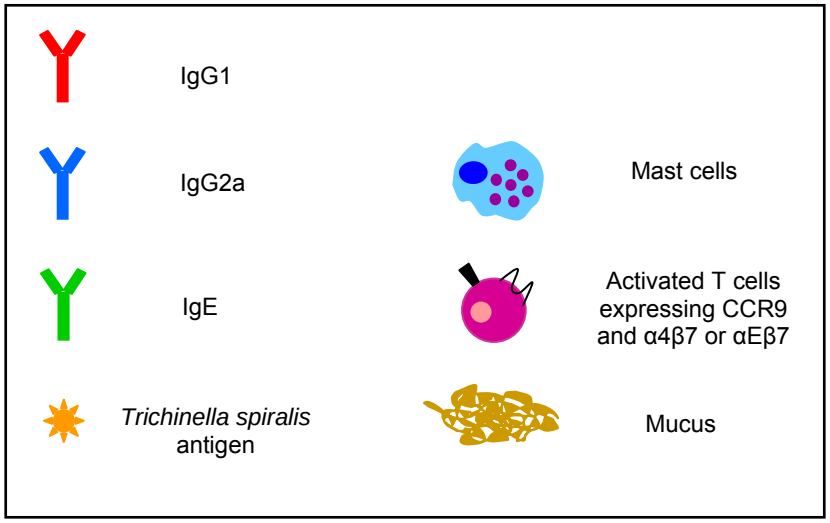
mast cells effect rapid expulsion in this model of protection. Isotypes induced by parenteral infection reflect a modified Th2 profile associated with chronic parasite infections (22). IgG1 and IgG2a were dominant with little IgE in parenterally infected rats, while IgG1 and IgE were elevated in orally infected rats. Because IgG1 was elevated in both models of infection, and because this isotype causes mucus entrapment in neonatal rats and *in vitro*, it is likely that this isotype mediated mucus entrapment in parenterally infected rats. IgE and IgG2a are either not essential, or they play compensatory roles in effecting rapid expulsion. To address this question, parenterally infected nude rats could be passively transferred with parasite specific IgG2a and IgE prior to challenge infection. In addition, complement was not essential for rapid expulsion in parenterally infected rats.

In the absence of inflammatory and architectural changes in the intestine, parenteral infection must induce more subtle alterations in the intestinal environment that promote the protective effects of antibodies. It is likely that muscle larvae residing in the tongue provide an antigenic stimulus at a mucosal site. T cells, primed at this location, may up-regulate homing molecules such as α4β7 and CCR9 or αEβ7 that direct migration to the intestine (10). The integrin α4β7 binds MadCAM-1 and is important for T cell entry into the lamina propria, while the chemokine receptor, CCR9 binds TECK and localizes T-cells to the small intestinal epithelium (20). The integrin αEβ7 is expressed on many mucosal T cells, and it ligand is E-cadherin. Alternatively, since parenteral immunization establishes a persistent muscle infection (60 days in our experiments), systemic activation of lymphocytes might also induce trafficking to mucosal sites. Activated T cells migrating to the intestine might then influence intestinal cells by producing cytokines.

While mast cell numbers are not increased, they become activated during expulsion. It is possible that the Th2 cytokines released by T-cells might alter the resident mast cell FcR expression to a higher A/I ratio making these cells more responsive to stimulation by either IgE or IgG2a immune complexes. RMCP-II release causes increased paracellular permeability in the intestine (19), and this would facilitate IgG1 transport to the lumen. Thus IgG1 provides protection by encumbrance of larvae within the intestinal epithelium. This model of protection induced by parenteral immunization is depicted in Figure 5.1. *T. spiralis* provides an excellent system to further investigate mucosal immunity induced by parenteral immunization.

Figure 5.1: Model of immunity induced by parenteral immunization. (1) T cells activated by muscle larvae residing in the tongue upregulate expression of α4β7 and CCR9. These cells migrate to the intestine where they release Th2 cytokines that influence resident mast cells. (2) Upon re-infection, mast cells, activated by either IgE or IgG2a complexed with parasite antigen, release RMCP-II. (3) RMCP-II increases intestinal paracellular permeability and aids translocation of parasite specific IgG1 to the gut. (4) IgG1 mediates mucus entrapment of larvae. (5) IgG1 encumbers larvae within the epithelium and blocks their sensory reception that impedes epithelial invasion.


Trichinella spiralis within the intestinal epithelium
1
2
3
4
5
Th2
Th2


IgG1
IgG2a
IgE
Trichinella spiralis antigen
Mast cells
Activated T cells expressing CCR9 and α4β7 or αEβ7
Mucus

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